Assessment of novel disinfection technologies, and bacterial contamination in the healthcare setting.

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A thesis presented to Ulster University for the degree of Doctor of Philosophy

November 2018

I confirm the word count of this thesis is less than 100,000 words

“Always make a total effort, even when the odds are against you”

Arnold Palmer

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List of Abbreviations

µg	Microgram
µL	Microlitre
µm	Micrometre
AMI	Antimicrobial technology
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
BHI	Brain heart infusion
CAUTI	Catheter-associated urinary tract infection
CDC	Centers for Disease Control and Prevention
CEN/EN	The European Committee for Standardization
CFU	Colony forming unit
CHEF	Contour-clamped homogenous electric field
CLABSI	Central line-associated bloodstream infections
cm	centimetre
CRE	Carbapenem-resistant *Enterobacteriaceae*
CSA	CHROMagarTM Staph aureus
DNA	Deoxyribonucleic acid
DSM/DSMZ	Leibniz-Institute DSMZ German Collection of Microorganisms and Cell Cultures
ECDC	European Centre for Disease Prevention and Control
EU	European Union
EUCAST	European union committee for antimicrobial susceptibility testing
g	Gram
GS	Goldshield
h	Hour
HAI	Healthcare acquired infection
HAP	Hospital-acquired pneumonia
HCAI	Healthcare acquired infection
HPV	Hydrogen peroxide vapour
ICU	Intensive care unit
L	Litre
MAR	Multiple Antibiotic Resistance Index
MDR	Multi-drug resistant
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
min	Minute
mL	Millilitre
MLST	Multi-Locus Sequence Typing
mM	Millimolar
mmol	Millimoles
MRSA	Methicillin-Resistant Staphylococcus aureus
MSSA	Methicillin-Sensitive Staphylococcus aureus
MVLST	Multi-virulence-locus sequence typing
NHS	National Health Service
NHSCT	Northern Health and Social Care Trust

NHSH	National Healthcare Safety Network
nm	Nanometre
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PFGE	Pulse-field Gel Electrophoresis
QAC	Quaternary Ammonium Compound
QMRA	Quantitative microbial risk assessment
RAPD	Random Amplification of Polymorphic DNA
rpm	Revolutions per minute
s	Second
SD	Standard deviation
SICU	Surgical intesive care unit
siQAC	Organosilane coupled with Quaternary Ammonium Compound
SSI	Surgical site infection
ST	Sequence type
TBE	Tris/Borate/EDTA
TESSy	The European Surveillance System
TSA	Typtone soya agar
TSB	Typtone soya broth
TVC	Total viable count
UK	United Kingdom
USA	United States of America
v	Volume
V	Voltage
VRE	Vancomycin-resistant Enterococcus
VRSA	Vancomycin-Resistant Staphylococcus aureus
w	Weight
WGS	Whole genome sequencing
WHO	World Health Organisation
WHSCT	Western Health and Social Care Trust

Declaration

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Jason Murray

Acknowledgments

A PhD is truly a team effort and I have so many people to thank. I would like to thank my supervisory team – Dr Nigel Ternan, Dr Chris Gill, Prof Geoff McMullan, Dr David Farren and Prof Mike Scott – for all their help, expertise and advice throughout my PhD journey. A special mention to Nigel and Chris, both of whom were always available no matter the issue (as much as my "Derryisms" annoyed them). I would also like to thank Prof James Dooley who was always willing to share his vast knowledge of all things microbiology. I would like to thank all the staff at Antrim Area Hospital who were very accommodating during my visits. A special mention for all the amazing technicians at Ulster for all you help.

To all the PhD students past and present who have been lucky enough to grace W0039 including Scott, Bader, Syed, Michael and many more. I've been told I tend to talk a lot so thanks for putting up with me – I truly consider you all friends and thank you. To all the post docs who gave me advice along the way – thank you Ciara my first post doc and Matt for the endless thesis writing tips and random chats about anything and everything. Thanks to Jagan and Michael – always dependable for a pint and a chat about all things sport.

A special mention for Zeus and the girls. Zeus has been a great friend and always willing to help me out no matter – I really appreciate it habibi. Collen (aka Dave), Mary, Lucy and Carla have been the best help in procrastinating from my thesis writing, from random Friends quotes to the best Gavin and Stacey impressions to gifs to drinks and balloons (so many balloons) – you have been more helpful than you know so thank you, I truly appreciate everything.

To all the fellas at Foyle Golf Centre, Derry especially the esteemed members of "Shit Golfers Inc."– I strongly believe you need a hobby away from your PhD and golf has been that for me and without you all it wouldn't be as much craic – so thanks to you all. And thanks to all my friends in Derry for everything and always being there.

To my family, thanks to my mum and dad for everything you have ever done for me and all the support and love you have shown me, I am so grateful for everything you have given me. To my brother Daniel and sisters Clare and Jacqueline – thanks for all your encouragement. And my niece Erin and nephews Shea, Caomhan, Luca and Jake. All of you guys mean so much to me. I would also like to thank Katie's family, Paul and Deirdre, you have done so much for me and I can't thank you enough.

And finally to my Katie, nobody will ever know how important to you are to me. I can say without doubt that without you I wouldn't have ever went back to uni or got to this stage in my PhD. You encouraged me to go back to my studies, and on days when I wanted to give up you were always there to give me the encouragement I needed. You will never understand how much you mean to me, I am so lucky to have ever met you. Love you.

Abstract

Worldwide, hundreds of thousands of healthcare acquired infections (HAIs) are reported each year. HAIs are infections that occur whilst in hospital or as a result of hospital admittance, contamination of hospitals is a source of, and allows dissemination of HAIs. In healthcare settings one of the major vectors of contamination is healthcare workers' uniforms. As surfaces become contaminated, bacteria can then be contacted by patients (direct transmission) or staff who may then indirectly spread those bacteria to patients. Both direct and indirect spread of bacteria could result in infection of patients ultimately resulting in increased infection rates and associated costs. A further consequence of such a scenario is the increased levels of antibiotic use, the survival of antibiotic resistant bacteria and increased prevalence of antimicrobial resistance.

A pilot study was conducted at Antrim Area Hospital, Northern Health and Social Care Trust. 100 pre-shift and 100 post-shift healthcare workers' uniforms were assessed for *Staphylococcus aureus* and *Enterococcus* spp. isolates. We found increased levels of antibiotic resistant *S. aureus* and antibiotic resistant *Enterococcus* spp. contamination on post-shift uniforms compared to zero to minimal contamination of pre-shift uniforms. A biobank of isolates was created and subsequently characterised for antibiotic sensitivity using European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines – 51% of *S. aureus* isolates were classed multi-drug resistant. Genomic diversity was assessed using Random Amplification of Polymorphic DNA (RAPD) – high levels of similarity was found amongst isolates. As one means of reducing uniform bioburden, we conducted analysis of a novel surface active organosilane disinfectant named Goldshield (GS). GS was marketed as a long lasting antimicrobial with residual activity to prevent (re)contamination. GS was tested against model HAI bacteria, spores and biofilms with a view to assessing its potential incorporation into infection control practices. GS technology displayed bactericidal, sporicidal and anti-biofilm properties in laboratory testing providing rationale for an intervention where GS could be incorporated into hospital laundry and assessed for potential use in infection control.

List of Publications, Conferences and Events

Publications

1. Murray, J., Muruko, T., Gill, C.I.R., Kearney, M.P., Farren, D., Scott, M., McMullan, G., Ternan, N.G. (2017) Evaluation of bactericidal and anti-biofilm properties of a novel surface-active organosilane biocide against healthcare associated pathogens and *Pseudomonas aeruginosa* biofilm. *PLOSOne.* 12: e0182624

Conferences

1. 7th Annual Translational Medicine (TMED7) Conference (2015)
   Poster Presentation – *Healthcare acquired infections: intervention strategies to prevent surface (re)contamination).*
2. Queens University Belfast Annual Infection and Immunity Meeting (2015)
   Poster Presentation – *Healthcare acquired infections: intervention strategies to prevent surface (re)contamination).*
3. Festival of PhD Research – Ulster University (2017)
   Oral Presentation – *Assessment of the reservoir potential of healthcare workers' uniforms as a source of antibiotic resistant pathogenic bacteria.*
4. Medical Research Foundation Antimicrobial Resistance Conference (Bristol) (2018)
   Poster Presentation – *Assessment of the reservoir potential of healthcare workers' uniforms as a source of antibiotic resistant pathogenic bacteria.*
5. Boxing clever against the superbugs – NI AMR (2018)
   Oral Presentation – Healthcare workers' uniforms: a source of multidrug resistant bacteria.

Training Events/Cohorts

1. Medical Research Foundation National PhD training programme in Antimicrobial resistance research. Attended residential training week and cohort member.

Chapter 1

General Introduction

1.1 Healthcare Acquired Infections

Healthcare acquired infections are often referred to as hospital acquired infections, 'superbugs' or nosocomial infections and are often abbreviated as HCAIs or HAIs [HAIs hereafter]. Cardoso *et al.* (2014) defined HAIs as "*a localized or systemic condition: 1) that results from adverse reaction to the presence of an infectious agent(s) or its toxin(s) and 2) that was present 48 hours or more after hospital admission and not incubating at hospital admission time*" –thus a HAI is an infection acquired from the healthcare environment as opposed to community/community associated infections (Cardoso *et al.*, 2014).

HAIs are categorised based on the nature of the infection, or the procedure under which the infection occurred. Common terms used to describe HAI classifications include central line-associated bloodstream infections (CLABSIs) (Pallotto *et al.*, 2017), catheter-associated urinary tract infections (CAUTIs) (Rebmann and Greene, 2010), surgical site infections (SSIs) (Lee, 2000), hospital-acquired pneumonias (HAP) (Leu *et al.*, 1989; Sprigings *et al.*, 2017) and gastrointestinal infections (predominantly caused by *Clostridium difficile* (Schmier *et al.*, 2016). A considerable diversity of microorganisms are responsible for HAIs, including *Staphylococcus aureus*, *Enterococcus* spp., *Clostridium difficile*, carbapenem-resistant *Enterobacteriaceae* (predominantly *Escherichia coli, Klebsiella* spp, *Enterobacter* spp.), *Acinetobacter* spp. and *Pseudomonas* spp (Dancer, 2014).

1.2 Implications of Healthcare Acquired Infections

1.2.1 Healthcare Acquired Infection Incidence Rates

HAIs are directly responsible for increased morbidity and mortality rates (Magill *et al.,* 2014; Zingg *et al.,* 2015). Worldwide it is estimated that hundreds of millions of patients are affected by HAIs yearly (Pittet *et al.*, 2017). In Europe 'The European Surveillance System' (TESSy) collects, analyses and reports data on communicable diseases including HAIs. However, issues exist in standards of reporting of HAIs and compliance in reporting varies per hospital and per country, and therefore most reported numbers tend to be estimates based on large sample groups. It is estimated that HAIs are the cause of 4.5 million infections each year in the European Union (EU) alone and that these result in approximately 37,000 deaths per year (Lamarsalle *et al.*, 2013; Zingg *et al.*, 2015; European Centre for Disease Prevention and Control, 2018). The most common type of HAI is a SSI (Al-Tawfiq and Tambyah, 2014), and the European Centre for Disease Prevention and Control (ECDC) reported that, within the EU in 2016, there were a total of 630,551 (14% of total HAI cases) surgical site HAIs (European Centre for Disease Prevention and Control, 2016). In the UK 198,138 (~4.5% of EU total) cases of HAI were reported from 365 hospitals; more locally in Northern Ireland a total of 10,288 HAI cases were reported from 10 hospitals (European Centre for Disease Prevention and Control, 2016).

In the USA there is no standalone surveillance system for HAI incidence and infection numbers published are national estimates based on sub-population sample groups. The 'US Centers for Disease Control and Prevention' (CDC) most recent estimation was ~1.7 million HAIs per year resulting in 35,967 deaths annually in the USA (Klevens *et al.*, 2007; Magill *et al.*, 2014; Scott, 2009). In 2014 Magill *et al.* conducted a large scale surveillance of infection

data across 10 US states in which they investigated acute care hospitals only – they concluded that there were 721,800 HAIs per year for 648,000 patients (Magill *et al.*, 2014). They also concluded "*on any given day approximately 1 of every 25 inpatients in U.S. acute care hospitals has at least one health care–associated infection*" (Magill *et al.*, 2014) – this figure remains the most updated statistic reported by the CDC (Centers for Disease Control and Prevention, 2018). The most common infections were pneumonias and SSIs, however the most common causative HAI organism was *C. difficile* (for all HAI infection types) (Magill *et al.*, 2014).

In regard to location within healthcare facilities, intensive care units (ICUs) are the most common place for HAIs to occur (Šuljagić *et al.*, 2005; Sadatsafavi *et al.*, 2016). The ECDC reported that 8.4% of patients (12,735 patients) staying in ICUs for more than 2 days experienced a HAI – in their reports the most common infections were *P. aeruginosa*, *S. aureus*, *Klebsiella* spp. and *E. coli* (European Centre for Disease Prevention and Control, 2016b). Moreover, Custovic *et al.* (2014) stated that is it 5-10 times more likely that HAIs will occur in ICUs compared to other hospital departments, and indeed it has been reported that 40-50% of ICU patients develop HAIs (Custovic *et al.*, 2014). Furthermore, a recent publication used molecular typing methods to robustly demonstrate transmission of HAIs around a hospital setting in Australia (Leong *et al.,* 2018).

Geography also influences HAI prevalence. Allegranzi *et al* (2011) conducted a meta-analysis on HAI prevalence, comparing developing countries to developed countries. In these countries from Africa, Americas, Europe, Southeast Asia, eastern Mediterranean and western pacific were included and classed developing or developed according to the WHO classification. They concluded that increased incidence of HAIs in developing countries was

due to lower budgets for infection control and prevention. They reported that 7.1% of all patients in Europe suffer a HAI and that 4.5% of USA patients suffer a HAI (Allegranzi *et al.*, 2011). However, in developing countries, 15.5% of patients suffer a HAI based on pooled information from high quality studies (Allegranzi *et al.*, 2011). It is likely that the increased incidence of HAIs in developing countries compared to developed countries is as a result of poorer hygiene and less money available for infection prevention.

1.2.2 Financial Burden of Healthcare Acquired Infections

The costs associated with HAIs are substantial. Associated expenses include costs attributable to increased length of stay in hospital (due to increased requirement of resources and staffing costs), increased diagnostic expenses to determine cause of infection and appropriate treatment, and increased treatment expenses once the HAI has been diagnosed. For example, during an outbreak of *K. pneumoniae* in USA, it was reported that the total cost due to 486 infections was $341,751 (Stone *et al.* 2003). These costs were broken down as healthcare workers time ($146,331 for additional staff time of 2489 h ($58 per hour)), surveillance and infection control procedures ($66,794 for 1055 h staff time ($63 per hour)), additional laboratory procedures ($56,716), and loss of bed space/lost revenue ($109,680). Additionally, the average increase for length of stay in hospital ranged from 25 to 181 days (average increase in length of stay was 48.5 days) (Stone *et al.*, 2003).

WHO estimates that the incidence of HAIs leads to an additional 16 million extra patient days in hospital in the EU (World Health Organisation, 2011; Zingg *et al.*, 2015), accumulating to an expenditure of €7 billion per year (World Health Organisation, 2011; Lamarsalle *et al.*, 2013). In the UK it is estimated that HAIs cost €54 million (~£47 million as per conversion

rate October 2018) per year, while in France alone, expenditure is equal to €130 million (~£113 billion as per conversion rate October 2018) (World Health Organisation, 2011). In the USA it is estimated that the overall cost of HAIs is $28 billion to $45 billion annually (Scott, 2009; Stone, 2009) and a single HAI case could cost between $962 and $44,204 depending on the type of infection, with pneumonia cases being the most expensive HAI cases (Scott, 2009; Eber *et al.*, 2010; Anderson *et al.*, 2013; Zimlichman *et al.*, 2013; Schmier *et al.*, 2016); shown in Table 1.1. Table 1.2 summarises HAI costs based on infectious agent.

Table 1.1 – Direct medical costs of different types of healthcare acquired infections (HAIs) in the USA.

Type of Healthcare Acquired Infection	**Lowest estimated cost per case ($)**	**Highest estimated cost per case ($)**
Catheter-associated urinary tract infection (CAUTI)	$962	$1167
Central line-associated bloodstream infection (CLABSI)	$8379	$49201
Gastrointestinal infection (GI)	$8531	$12119
Surgical site infection (SSI)	$14572	$40688
Ventilator-associated pneumonia (VAP)/Hospital-acquired pneumonia (HAP)	$19475	$43112

Figures representative of estimated cost of healthcare acquired infection types in the USA. Information collated from various sources (Scott, 2009; Eber *et al.*, 2010; Anderson *et al.*, 2013; Zimlichman *et al.*, 2013; Schmier *et al.*, 2016).

Table 1.2 – Costs and length of hospital stay for outbreaks of individual healthcare acquired infections due to different bacterial pathogens.

Bacteria	**Number of infections**	**Increased length of stay per case**	**Overall Increased costs**	**Source**
Klebsiella pnuemoniae	486	48.5 days (mean)	$341,751 (total)	(Stone *et al.*, 2003)
Acinetobacter baumannii	34	11 days (mean)	$98,575 (total)	(Wilson *et al.*, 2004)
Enterococcus spp	277	17 days (mean)	$77,558 (mean per case)	(Song *et al.*, 2003)
Pseudomonas aeruginosa	82	20 days (median)	Not reported	(Carmeli *et al.*, 2006)
Staphylococcus aureus	348	8 days (mean)	$22,818 (mean per case)	(Cosgrove *et al.*, 2005)
Clostridium difficile	40	3.6 days (mean)	$3669 (mean per case)	(Kyne *et al.*, 2002)

The costs discussed above are primarily limited to assessments of direct care cost, i.e. hospital/treatment based cost (Marchetti and Rossiter, 2013). However a social impact is also observed as result of HAIs, with the main societal cost of HAIs being a loss of earnings due to increased length of stay in hospital. Marchetti and Rossiter (2013) assessed the cost of HAIs in USA from both clinical and societal perspectives. They concluded that when social costs are included, the 'true' total cost of HAIs per year in the USA rises from $28 billion to $45 billion (Scott, 2009) (when only direct costs are considered) to $96 billion to $147 billion (Marchetti and Rossiter, 2013).

1.2.3 Healthcare Acquired Infections and Antimicrobial Resistance

The development of antimicrobial resistance is a multifactorial problem that arises due to the overuse and misuse of antibiotics (O'Neill, 2016). However, the prevalence of HAIs also contributes to the emergence of antimicrobial resistance in pathogenic bacteria associated with HAIs (Holmes *et al.*, 2016; Swaminathan *et al.*, 2017). Thus antimicrobial resistance promotes the persistence and prevalence of HAIs (O'Neill, 2016). The well-established relationship between HAIs and antimicrobial resistance is evidenced by the development of national surveillance programmes, and the publication of comprehensive government reports on the topic. In the USA, the CDC and National Healthcare Safety Network (NHSH) publish the *"Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention,"* which every three years, reviews antimicrobial resistance patterns amongst HAIs, collating incidence data and antimicrobial resistance data together (Hidron *et al.*, 2008; Sievert *et al.*, 2013; Weiner *et al.*, 2016). In the UK Jim O'Neill

recently published a series of reports detailing the issue of antimicrobial resistance, included was a report dedicated to the role of HAIs entitled "*Infection prevention, control and surveillance: limiting the development and spread of drug resistance*" (O'Neill, 2016). The overall message of this report was that by improving infection prevention practices, infection rates would be reduced which would ultimately reduce antimicrobial resistance.

Antimicrobial resistance reduces the effectiveness of antibiotics against HAIs resulting in longer infections, leading to increased morbidity and mortality rates in hospitals (Cardoso *et al*, 2014; Al-Taani *et al*, 2018). Antibiotic-resistant HAIs commonly occur in hospitals (Cardoso *et al.*, 2014b; Ventola, 2015) and it is estimated that 30-40% of patients in European hospitals are receiving antibiotic therapy (European Centre for Disease Prevention and Control, 2013) – this combination results in a major driver of antimicrobial resistance (Holmes et al., 2016). It is also estimated that 16% of the bacteria causing HAIs are antimicrobial resistant (Hidron *et al.*, 2008). In 2008 it was estimated that half of the deaths caused by HAIs in Europe were caused by multi-drug resistant bacteria (Watson, 2008; ter Meuken, 2009). In summary HAIs are often antimicrobial resistant pathogens – making them more difficult to treat as certain antibiotics could be ineffective against them. There is also a consequence of increased antibiotic use and subsequently increased antimicrobial resistance.

Furthermore, extra expense is incurred when infections due to antimicrobial resistant bacteria occur. This is due to the added expense/time to diagnose antimicrobial resistance and determine a suitable antibiotic for treatment (Stone, 2009; Cheng *et al.*, 2015). It is reported that antimicrobial resistance results in greater length of stay and higher costs for drug-resistant infections compared to non-resistant infections – evidence of this has been

reported for beta-lactam resistant *K. pneumoniae* (Stone *et al.*, 2003), multi-drug resistant *A. baumannii* (Wilson *et al.*, 2004), vancomycin resistant *Enterococcus* spp. (Song *et al.*, 2003), multi-drug resistant *P. aeruginosa* (Carmeli *et al.*, 2006), methicillin resistant *S. aureus* (Cosgrove *et al.*, 2005) and *C. difficile* (Kyne *et al.*, 2002) – information regarding increased costs and length of stay is summarised in Table 1.2 It has also been reported that a drug resistant case of *S. aureus* costs twice as much to treat as a drug-sensitive infection ($16,000 vs. $35,000) (Filice *et al.*, 2010; O'Neill, 2016). Similarly, treatment of penicillin-resistant *Streptococcus pneumoniae* was more expensive that a penicillin-susceptible *S. pneumoniae* (211 $CDN vs. 74 $CDN) (Quach *et al.*, 2002; Cheng *et al.*, 2015).

In conclusion HAIs lead to increased infection rates leading to increased antibiotic use which contributes to increased antimicrobial resistance (O'Neill, 2016). Struelens (1998) summarised the cycle – *"Resistance* (to antimicrobials) *results from the interplay of micro-organisms, patients, and the hospital environment, including antibiotic use and infection control practices"* (Struelens, 1998) – i.e. this is a recurring cycle with an interplay of associated factors. As these factors are intrinsically linked, reducing one could allow a reduction in others, for example reducing HAIs prevalence could lead to subsequent reduction in antimicrobial resistance (O'Neill, 2016).

1.3 Contamination of the Healthcare Environment

It has been shown that many factors contribute to the prevalence of HAIs in hospitals, including increased antibiotic use (Holmes *et al.*, 2016), the susceptibility of patients increasing their likelihood of catching an infection and critically, poor infection control compliance (Lee *et al.*, 2018). However, after much debate and conflicting reports (Malik *et al.*, 2003) it is now accepted that microbial contamination of the healthcare environment is a significant contributing factor in the emergence, spread and prevalence of HAIs (Mitchell *et al.*, 2013; Dancer, 2014; Doll *et al.*, 2018; Lee *et al.*, 2018).

Microbial contamination of the hospital environment is responsible for direct and indirect transmission of microorganisms to patients (Martínez *et al.*, 2003; French *et al.*, 2004; Dancer, 2014; Cheng *et al.*, 2015). When the environment becomes contaminated with pathogenic organisms, those objects/surfaces/people act as a source of infectious agents (Kramer *et al.*, 200*6;* Cheng *et al*, 2015). High touch surfaces are the most problematic as they are the most likely to become contaminated with, and harbour, potentially pathogenic microorganisms, and to act as vectors for transmission to vulnerable patients (Dancer, 2014; Cheng *et al.*, 2015). Examples of high touch surfaces include bed rails, door handles (Muirhead *et al.*, 2017), bed clothing (mattresses/sheets) (Tarrant *et al.*, 2018), staff and staff equipment/uniforms. The routes of environmental transmission of HAIs is presented in Figure 1.1 (Cheng *et al.*, 2015).

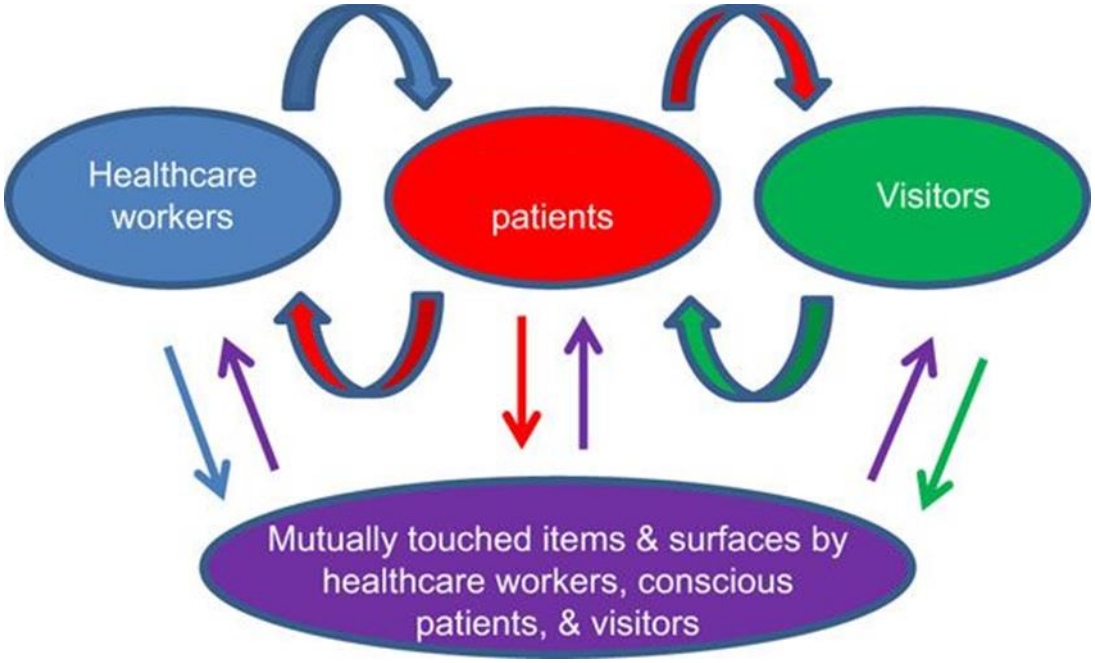

Figure 1.1 – The routes of environmental transmission of HAIs. When high-touch surfaces become contaminated they can contribute to the spread of healthcare acquired infections via direct (patient) and indirect transmission routes (others) – arrows represent transmission of environmental pathogens (Cheng *et al.*, 2015).

In the early 1990s cleaning was considered non-critical in infection prevention practices; resulting in a misguided a cost-saving scheme in the UK NHS in the 1990s in which financial support for cleaning was reduced (Dancer, 1999; Dharan *et al.*, 1999). This correlated with a subsequent rapid increase in HAIs prevalence in the late 1990s to early 2000s with resulting increased interest from health service providers, industry and researchers alike (Dancer, 2014). As of 2018, cleaning and decontamination for infection prevention purposes is considered vital and therefore interest in methods for decontamination of the healthcare environment has increased across a range of sectors and disciplines.

The environmental contamination of high touch surfaces in healthcare settings by methicillin-resistant *S. aureus* (MRSA) was first described in 1997 by Boyce *et al.* (1997) – this study demonstrated that healthcare workers' gloves became contaminated with MRSA due

to their touching contaminated sites/objects (including patient bedside furniture) (Boyce *et al.*, 1997). Evidence of hospital contamination by pathogenic bacteria can be found throughout the literature including by MRSA (Knelson *et al.*, 2014; Lin *et al.*, 2017), vancomycin-resistant *Enterococcus* (VRE) (Bonten *et al.*, 1996; Knelson *et al.*, 2014), carbapenem-resistant *Enterobacteriaceae* (CRE) (Lerner *et al.*, 2013; Weber *et al.*, 2015), *C. difficile* (Weber *et al.*, 2010; Sitzlar *et al.*, 2013) and *Acinetobacter* species (Weber *et al.*, 2010). A recent publication (Deshpande *et al.*, 2017) described contamination of hospital floors with *C. difficile*, MRSA and VRE – and the authors concluded that this contamination leads to further contamination of high touch objects/surfaces. In addition, work by other researchers has determined the survival periods of known HAI agents on inanimate surfaces representative of surfaces found in healthcare settings where bacteria can survive for up to one and a half years (Table 1.3).

Table 1.3 – Survival times of known healthcare acquired infection-causing organisms and infectious doses.

Organism	**Reported survival time**	**Infectious dose**
Methicillin-resistant *Staphylococcus aureus*	7 days to 7 months	4 CFU
Acinetobacter	3 days to 5 months	250 CFU
Clostridium difficile	>5 months	5 spores
Vancomycin-resistant *Enterococcus*	5 days to 4 months	$<10^3$ CFU
Escherichia coli	2 hours to 16 months	10^2-10^5 CFU
Klebsiella	2 hours to 30 months	10^2 CFU
Norovirus	8 hours to 7 days	<20 virons

*table taken directly from Dancer (2014) who extrapolated information from published materials as part of a review (Wandall, Arpi and Wandall, 1997; Kjerulf *et al*, 1998; Kramer, Schwebke and Kampf, 2006; Makison and Swan, 2006; Eaton *et al*, 2008; Teunis *et al*, 2008; Chiang *et al* 2009; Lawley *et al*, 2010; Dancer, 2014).

Studies have also demonstrated the role that contamination plays in increasing incidence rates of HAIs (this is often shown inversely, i.e. reducing the bioburden reduces incidence). In an intervention to reduce environmental and staff contamination, Simmons *et al.* (2013) implemented hand hygiene and novel disinfection systems over a 6 month period in a US hospital system and reported an average 56% reduction in infection rates of MRSA during the intervention period. Another example from a Glasgow teaching hospital assessed bioburden levels in a surgical intensive care unit (SICU) correlating these levels with HAIs rates (White *et al.*, 2008). They found that 25% of their samples (hospital beds, cardiac monitor buttons, curtains, keyboards and chairs) were contaminated with greater than 2.5 CFU/cm^2 *S. aureus*, meaning that 25% of the samples were deemed hygiene failures. They also reported that levels of contamination increased when occupation of the SICU increased and concluded overall that hygiene failures correlated with HAI levels, in that increased contamination resulted in increased HAI incidence rates (Figure 1.2) (White *et al.*, 2008).

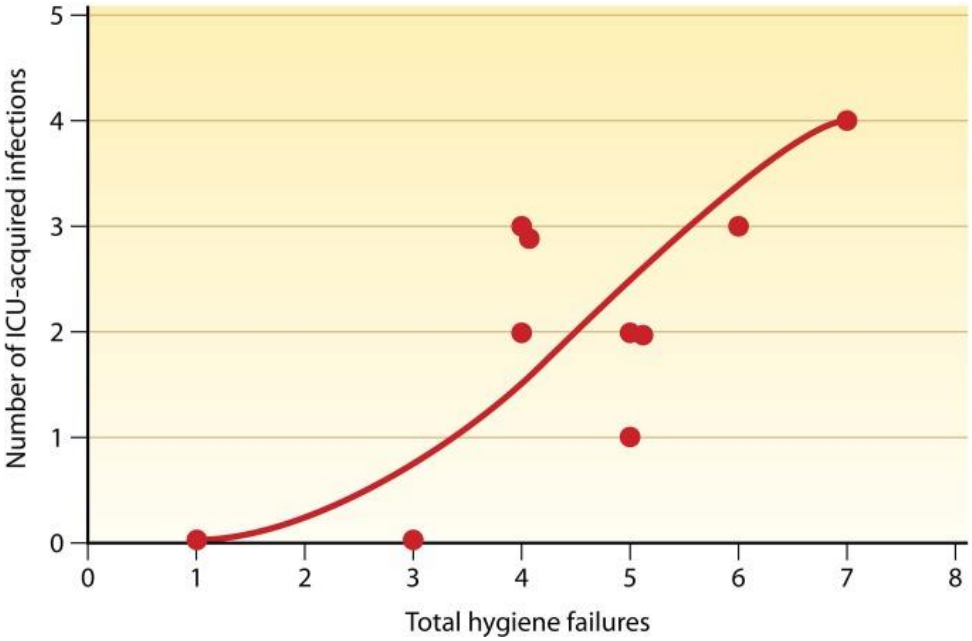

Figure 1.2 – Relationship between environmental contamination of a surgical intensive care unit and number of healthcare acquired infections during a 2 month sampling period (White *et al.*, 2008; Dancer, 2014).

1.4 Decontamination

Decontamination of the healthcare environment to remove pathogens is critical in reducing bioburden and thus the risk of HAIs (Han *et al.*, 2015). Appropriate decontamination will eradicate/prevent contamination of the healthcare environment and thus reduce spread of HAIs. Decontamination may be defined as a multi-purpose process whereby items are made clear of dirt and microorganisms. There are various levels of decontamination that may be employed depending on the purpose. For example the Western Health and Social Care Trust (WHSCT) define decontamination as a "combination of cleaning, disinfection and sterilisation" (WHSCT, 2015). Cleaning is defined as the process where a low grade detergent is applied to remove dust, oil and dirt from objects and eradicates a proportion of microorganisms. Disinfection is considered an intermediate grade decontamination process; this can be heat or chemical application aimed to reduce the bio-burden to a non-harmful level but doesn't eradicate all microorganisms and it does not destroy bacterial spores. Sterilisation however, is the complete removal of all microorganisms including bacterial spores, and is achieved mainly by the use of chemicals, and is common practice for surgical instruments (WHSCT, 2015).

Current decontamination strategies have been shown to be 'unsatisfactory' throughout the published literature. One such study in a London teaching hospital assessed 124 swab samples from high-touch points in surgical wards before and after cleaning (the cleaning process was not fully defined but detergent sanitizer and laundry processes were employed). They reported that 90% of the contaminated surfaces (before disinfection) remained contaminated (post disinfection) (French *et al.*, 2004). Many other examples of environmental contamination of pathogenic bacteria have been reported (Bonten *et al.*,

1996; Boyce *et al.*, 1997; Weber *et al.*, 2010, 2015; Lerner *et al.*, 2013; Sitzlar *et al.*, 2013; Knelson *et al.*, 2014; Deshpande *et al.*, 2017; Lin *et al.*, 2017).

1.4.1 Prevention of Contamination

Whilst reducing contamination of the healthcare environment is essential, prevention of contamination in the first place is the ideal scenario. As discussed, there is supporting evidence for contamination of the healthcare environment which can be transmitted to susceptible patients. Furthermore, there is evidence of re-contamination of the environment post decontamination. For example, Aldeyab *et al.* (2009) reported the re-contamination of successfully cleaned areas within 1 h of disinfection in Antrim Area Hospital. Similarly Attaway *et al.* (2012) assessed bacterial bioburden of hospital surfaces pre-disinfection and post-disinfection in a South Carolina hospital. They reported re-contamination levels as high as 45% of the pre-disinfection bioburden levels (i.e. prior to cleaning) after 2.5 h (Attaway *et al.*, 2012).

Some current methods of decontamination claim to prevent (re)contamination of the environment by their mode of action (Dancer, 2014). Examples of these include antimicrobial surfaces (for example antiadhesive surfaces) to prevent contamination and antimicrobial coatings (for example copper) which provide residual (long-lasting) antimicrobial activity (Baxa *et al.*, 2011; Dancer, 2014; Perez *et al.*, 2015). Such methods are discussed further in chapter 2.

1.5 Overview of Thesis

The main focus of this thesis is assessment of contamination of the healthcare environment and a novel approach to prevent (re)contamination of the healthcare environment. Working alongside an industrial partner and clinical collaborators a surface-active, long-lasting disinfection product was assessed *in vitro*. The main focus of this was to determine both antimicrobial activity and the residual activity of the product with a view to incorporating it in infection control practice in the Northern Health and Social Care Trust (NHSCT). This was to be in the laundry of healthcare workers uniforms to prevent contamination.

To demonstrate the need for such an intervention, the current bioburden status of healthcare workers uniforms was assessed in Antrim Area Hospital, Northern Health and Social Care Trust. Working with the consultant microbiologist and domestic services team, healthcare workers' uniforms were assessed for bacterial contamination pre-shift and post-shift. We hypothesised that this would demonstrate environment contamination and contamination of uniforms representing direct and indirect transmission routes of HAIs onto patients and provide rationale that steps are required to reduce the contamination of healthcare workers uniforms.

Chapter 2

Evaluation of bactericidal, anti-biofilm and sporicidal properties of a novel, surface-active organosilane biocide

Associated publication:

Murray, J., Muruko, T., Gill, C.I.R., Kearney, M.P., Farren, D., Scott, M.G., McMullan, G., Ternan, N.G. (2017) Evaluation of bactericidal and anti-biofilm properties of a novel surface-active organosilane biocide against healthcare associated pathogens and *Pseudomonas aeruginosa* biolfilm. *PLoS One* **12**: e0182624.

2.1 Introduction

There is a requirement for alternative technologies/compounds for decontamination of the healthcare environment. One alternative approach to tackle microbial contamination and re-contamination is the creation and use of antimicrobial surfaces, either by use of antimicrobial coating materials or via antimicrobial impregnated surfaces (Boyce, 2016).

2.1.1 Organosilanes

Organosilanes are a group of chemicals which can be used to create these antimicrobial surfaces. Silanes are chemicals containing a silicon-carbon bond. Silanes are monomeric, meaning functional groups can be formed by covalent bonds of reactive components to silanes (Gkana *et al.,* 2017). Organosilanes are molecules where two different reactive groups are bond with silanes. One of these reactive groups allows organosilanes to form strong covalent bonds to inorganic substrates which is effectively the coating system of these compounds (Gkana *et al.,* 2017). Organosilanes can then be coupled with an antimicrobial compound (often quaternary ammonium compounds). This combination has risen to application in disinfectants to provide residual protection (Baxa *et al.*, 2011; Boyce, 2016). Only a few studies have assessed the residual activity of these compounds with conflicting results and conclusions on the potential benefits of using these compounds in disinfection practices (Baxa *et al*, 2011; Boyce *et al*, 2014; Tamimi *et al*, 2014).

2.1.2 Goldshield

Goldshield (distributed by Goldshield technologies Ltd. [GS hereinafter]) is a patented, water soluble organosilane, coupled with a quaternary ammonium compound that is designed to

coat surfaces with a protective antimicrobial layer to prevent microbial contamination. The product was originally designed at Emory University, USA and is the subject of three US patents (patent nos. US5,959,014, US6,221,944, and US6,632,805) (Baxa *et al.*, 2011; Perez *et al.*, 2015).

GS products are marketed as water-based, non-toxic, non-leaching, environmentally-benign nano-molecular-assembly technology (Baxa *et al.*, 2011; Perez *et al.*, 2015). GS technology utilises a chemical structure that is marketed as bactericidal, virucidal and fungicidal. The sophisticated technology has a very specific mechanism for direct killing of microbes (Baxa *et al.*, 2011; Perez *et al.*, 2015). The oxygen–organosilane carrier complex allows attachment to almost any surface and/or textile and affixation creating a semi-permanent covalent bond. Nitrogen creates a positive charge hence attracting negatively charged microbes. The unique molecular assembly of a long carbon chain physically attacks the cell, penetrating the cytoplasmic membrane resulting, the company states, in proteins becoming denatured and bacterial cell lysis resulting in an outpour of intracellular materials. The process, described as a “molecular bed of nails” results in the death of microorganisms as they contact the GS coated surfaces. Figure 2.1 shows an outline of the chemical make-up of GS and Figure 2.2 details GS mode of action.

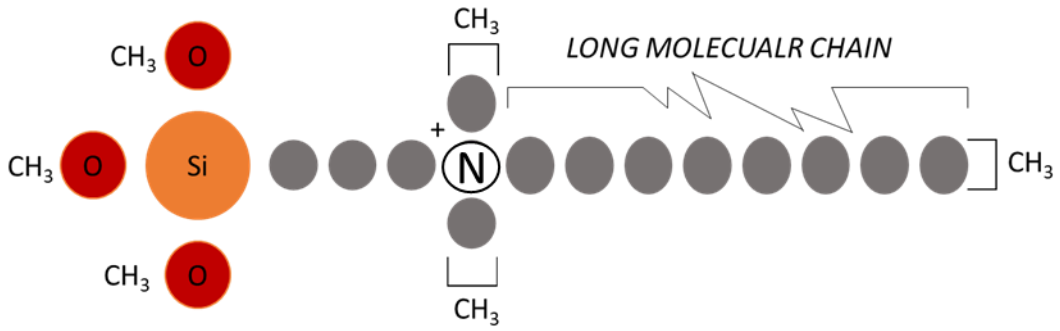

Figure 2.1 – Core Goldshield technology structure consisting of an oxygen-silane complex (for coating of surfaces), a nitrogen component (for attraction of microorganisms) and a long carbon chain (for physical disruption of microorganisms).

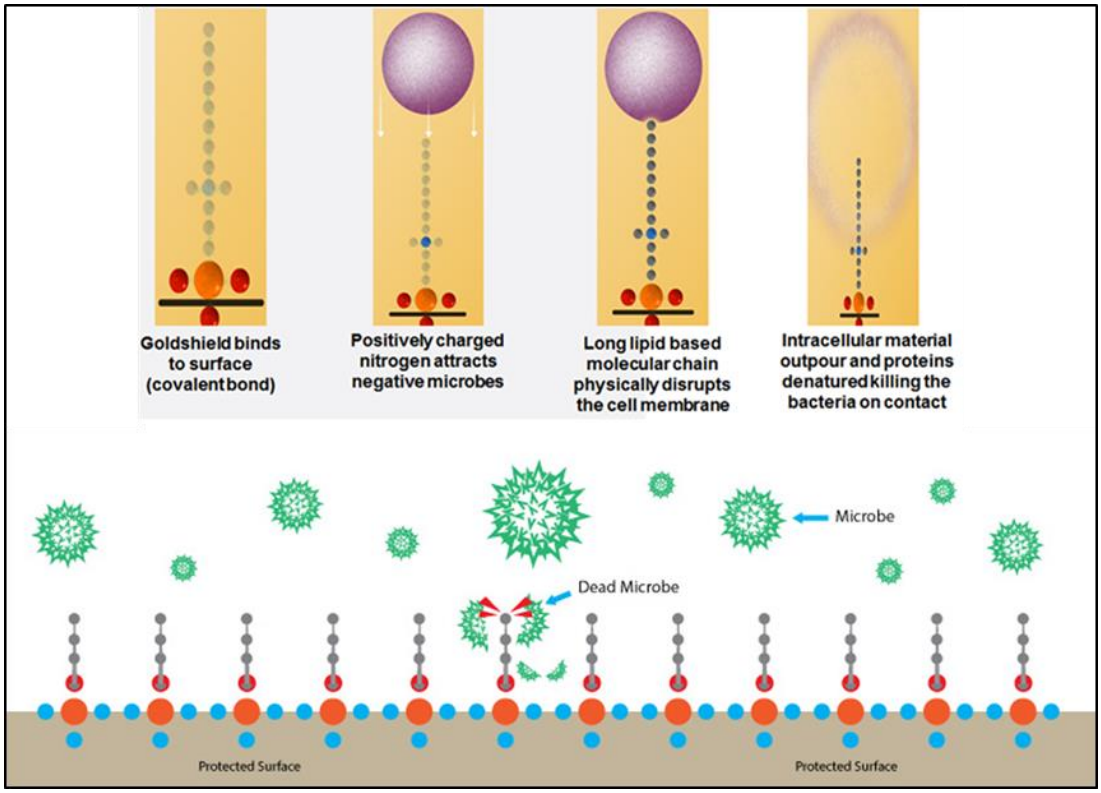

Figure 2.2 – Model for Goldshield mode of action. The process of how Goldshield acts as a surface-active protectant to kill any bacteria which contact the surface which could be an ideal solution in preventing contamination of hospital surfaces. GS coats surfaces forming a covalent bond. As microbes contact the surface they are attracted to GS via electrostatic charges. The long carbon chain physically ruptures microbial cell membranes resulting in a compromised membrane and ultimately cell death.

In 2014, GS had a range of products on the market based on the original technology developed at Emory. The products can potentially be used in a range of sectors, ranging from healthcare to schools, sport facilities, hospitality and military. GS5 (surface disinfectant) is the core product containing 3-trihydroxysilyl propyldimethyloctadecyl ammonium chloride (5%); Alcohols C12-16, Ethoxylated (1-5%); 2-butoxyethanol (1-5%); Pentaerythritol (<2%); and d-Limonene (<0.25%). Sporicidal products GS48 and GS48-55 are the same formulation as GS5, but with the addition of varying concentrations of hydrogen peroxide.

Peer reviewed, robust scientific research on Goldshield is limited to only two papers (Baxa *et al.*, 2011; Perez *et al.*, 2015). Baxa *et al.* (2011) assessed the effectiveness of the GS5 against *S. aureus* (MRSA), *P. aeruginosa*, *and E. coli* on fabrics, stainless steel and Formica surfaces. GS5 residual activity on fabrics was also tested over a 14 day period. Microbial bioburden Log_{10} reductions on treated surfaces were observed for stainless steel and Formica with 0.6 Log_{10} to 2.2 Log_{10} reduction dependent upon surface (Baxa *et al.*, 2011).

Perez *et al.* (2015) completed a hospital intervention study in which they used GS5 in University School of Medicine in Dearborn, Michigan. They determined the quantitative microbial risk assessment (QMRA) (which is a method of estimating risk of infection by exposure to microorganisms) both before and after GS5 use in environmental cleaning of high-touch surfaces in patient rooms. They documented reduction of bacterial bioburden of these surfaces and concluded that GS5 could prevent 5-10% of HAIs (Perez *et al.*, 2015).

2.1.3 Aims and Hypothesis

The aim of the work presented in this chapter was to assess the efficacy of GS technologies/products as disinfectant agents against a range of known HAIs. GS5 was initially tested as a standard disinfectant using suspension assays to directly assess antibacterial properties. The GS5 product was then tested as long-lasting disinfectant in comparison with competitor products to quantify residual antibacterial activity post application. GS5 was then tested to determine its residual activity against 10 known HAIs. Anti-biofilm activity was also assessed, by challenging *P. aeruginosa* DSM3227 biofilm formation with GS5. Finally, GS48 and GS48-55 products were tested against *C. difficile* spores. We hypothesised that GS technologies would exhibit bactericidal, anti-biofilm and sporicidal properties. It was also hypothesised that GS5 would exhibit residual antimicrobial activity.

2.2 Materials and Methods

2.2.1 Chemicals, Glassware and Media

All glassware was sterilised by soaking overnight in 1% Virkon (Antec, UK) and steam sterilised in an autoclave prior to use. All culture media (Oxoid, UK) was prepared as per the manufacturer's instructions. Phosphate Buffered Saline (PBS) (Oxoid, UK) was prepared in deionised water and steam sterilised in an autoclave prior to use. Two model surfaces were used. 316l Steel (Aalco, UK) and Formica. Sample of these materials were cut into 2cm×2cm chips, autoclaved (121 °C for 15 mins) and stored in a sealed sterile container prior to use in experiments.

2.2.2 Microorganisms

Ten bacterial species were obtained from either the American Type Culture Collection (ATCC) or the Leibniz-Institute DSMZ German Collection of Microorganisms and Cell Cultures (DSMZ). Bacteria included *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* DSM16358, *Mycobacterium smegmatis* DSM43469, *Pseudomonas aeruginosa* DSM3227, *Staphylococcus aureus* (MRSA) ATCC43300, *Staphylococcus aureus* (non-MRSA) DSM20231, *Staphylococcus epidermidis* DSM28319 (all cultured at 37 °C using Nutrient broth/agar), *Enterococcus faecalis* DSM12956 (37 °C using Tryptone soya broth/agar), *Burkholderia multivorans* DSM13243 (28°C using Nutrient broth/agar) and *Acinetobacter baumannii* DSM30008 (30 °C using Nutrient broth and agar). These were chosen as representative organisms of the type causing HAIs commonly seen in hospitals (U.S. Centers for Disease Control and Prevention, 2014) and included Gram positive organisms, Gram negative organisms and *Mycobacteria*. *Mycobacterium smegmatis* was used as it is a fasting-growing model *Mycobacterium* species

(Altaf *et al.*, 2010). Organisms were stored on Cryobeads (Technical Service Consultants Ltd, UK) at -80 °C and recovered in suitable media when required.

2.2.3 Disinfectant Agents

Five disinfectant agents were used (GS5, GS48, GS48-55, Actichlor and Distel). The characteristics of these antimicrobial agents are summarised in Table 2.1. Agents were acquired as full strength concentrate and working stock concentrations were prepared by dilution with deionised water as per the respective manufacturer's instructions.

Table 2.1 – Disinfectant products used in residual bactericidal testing.

Agent	Type	Active ingredient(s)	Concentration used*
Goldshield5	Organosilane coupled with Quaternary Ammonium Compound (siQAC)	3-trihydroxysilyl propyldimethyloctadecyl ammonium chloride	1:20 dilution (5%)
Goldshield48	Organosilane coupled with Quaternary Ammonium Compound (siQAC)	3-trihydroxysilyl propyldimethyloctadecyl ammonium chloride Hydrogen peroxide	1:20 dilution (5%)
Goldshield48-55	Organosilane coupled with Quaternary Ammonium Compound (siQAC)	3-trihydroxysilyl propyldimethyloctadecyl ammonium chloride	1:20 dilution (5%)
Actichlor	Chlorine-based disinfectant	Sodium dichloroisocyanurate	1:10 dilution (10%)
Distel	Quaternary Ammonium Compound	Tertiary amine and quaternary ammonium compounds	1:100 dilution (1%)

* as per manufacturer's instructions.

2.2.4 Direct Bactericidal Assessment of GS5

To determine the direct bactericidal activity of GS5, a suspension contact time assay was completed; varying concentrations of GS5 and *S. aureus* ATCC43300 were mixed followed by recovery and enumeration of viable cells to determine Log_{10} reduction. 0% (sterile water), 0.25% (v/v), 0.5% (v/v) and 1% (v/v) GS5 dilutions were prepared using sterile water as diluent. A 10 µL aliquot of mid-log phase *S. aureus* ATCC43300 was mixed with each of the GS5 concentrations and left to stand at room temperature for 5 min. Bacteria were enumerated by plating onto Nutrient agar and incubating at 37 °C for 24 h followed by direct colony counts.

2.2.5 Growth Calibration

For accurate application of precise bacterial numbers onto test surfaces, growth calibration testing was completed to determine the D_{600nm} to CFU/ml relationship for each organism. From an overnight culture a fresh culture was grown to a pre-defined D_{600nm} (D_{600nm} = 0.8-1). From this a range of serial dilutions (0% (broth reference), 20%, 40%, 60%, 80% and 100% of sample) were made using fresh broth. For each dilution D_{600nm} measurements were taken and CFU/mL determined by serial dilution and plated onto a suitable agar medium followed by incubation using appropriate incubation parameters. Following incubation colonies were enumerated, and CFU/mL determined. Data analysis (determination of *y intercept (y=*)) was conducted using Graphpad prism.

2.2.6 Residual Surface Activity of Disinfectants

To investigate the residual activity of surface disinfectants a protocol was developed from the EN13697 standard and the work of Baxa *et al*. (2011). *S. aureus* ATCC43300 (MRSA) and 316l Steel were used. The 316l Steel surface samples were sprayed with either GS5, Actichlor, Distel or sterile water (no treatment control) using a hand spray. The test surfaces were left to dry in the sterile environment of a category 2 cabinet (Biomat). *S. aureus* ATCC43300 was grown to mid-log phase of growth (D_{600nm} = 0.49) and diluted 1/100 using sterile PBS (Oxoid, UK). A total of 100 µL of this was added (in 10 µL droplets) to each surface. Bacteria were left on the surfaces for 45 min, and viable cells were then recovered in 10 mL of PBS by vortexing for 2 min. Viable bacteria were enumerated by plating on Nutrient Agar and incubating at 37 °C for 24 h followed by direct colony counts (Baxa *et al.*, 2011). Following recovery of bacteria from the surfaces, each surface was individually washed using sterile PBS, air dried and then stored in a sterile storage box. The surfaces were subsequently re-challenged with *S. aureus* ATCC43300 as described. This re-challenge was repeated at 3-day intervals over 15 days.

2.2.7 GS5 Bactericidal Surface Testing

A selection of 10 different bacteria, representative of important HAI bacteria, were individually tested on 316l Steel and Formica. Testing was performed to determine the maximum antimicrobial effect for a freshly treated surface. The protocol was as described above, but without re-challenge and only the activity of GS5 was assessed.

2.2.8 Assessment of GS5 Anti-biofilm Efficacy

P. aeruginosa DSM3227 biofilms were grown in 24-well microtiter plates and these were stained with 0.1% crystal violet to assess the extent of biofilm growth (Djordjevic *et al.*, 2002; Welch *et al.*, 2012; Shen *et al.*, 2013). To determine efficacy of GS5 against biofilm, microtiter plates (Thermo Scientific, UK) were pre-treated (prior to inoculation) with either 5% GS5 or sterile water (untreated): wells were soaked with 1 mL of agent for 10 min following which the treatment agents were aspirated and the plates left to dry in a sterile environment (Biomat category 2 cabinet). An overnight culture of *P. aeruginosa* DSM3227 was diluted 1/100 (using sterile nutrient broth) and microtitre plate wells were inoculated with a 1 mL aliquot of this, following which the plates were incubated aerobically at 37 °C. At defined time points (8 h, 12 h, 24 h, 48 h, 72 h and 96 h) biofilm production was assessed. The medium containing planktonic cells was removed and wells stained with 1.5 mL 0.1% Crystal Violet (Sigma-Aldrich, UK) for 10 min at room temperature. Unbound crystal violet (Sigma-Aldrich, UK) was removed and the stained wells were washed twice with 2 mL sterile PBS, following which the bound crystal violet was solubilised with 1.5 mL of 30% Acetic Acid (Thermo Scientific, UK) for 30 min at room temperature. A 1 mL aliquot from each well was transferred to a fresh 24-well microtiter plate and the absorbance of the crystal violet measured at 570nm (A_{5700nm}) using a FLUROstar Omega plate reader (BMG LABTECH, Europe).

2.2.9 Assessment of GS5 Effects on Bacterial Viability in Biofilm

Bacterial viability within biofilms was assessed using the BacLight Live/Dead bacterial viability kit (L-7007; Molecular Probes, Eugene, OR) (Webb *et al.*, 2003; Bauer *et al.*, 2013). With Baclight, live cells stain green and dead/damaged cells stain red. A stock solution was

prepared by mixing 4 µL of component A (1.67 mM Syto9 plus 1.67 mM propidium iodide), 6 µL of component B (1.67 mM syto9 plus 18.3 mM propidium iodide) and 1 mL of sterile water as described by (Webb *et al.*, 2003). *P. aeruginosa* DSM3227 biofilm was grown in 4-well Nunc™ Lab-Tek™ II Chamber Slide™ Systems (Thermo Scientific, UK) pre-treated with either 5% GS5 or sterile deionised water. Slides were inoculated with 1 mL of a 1/100 dilution of overnight culture of *P. aeruginosa* as above and incubated aerobically for 24 h and 48 h at 37 °C. At each time point during the experiment, excess media and planktonic cells were removed and the wells washed with sterile PBS followed by staining with 200 µL BacLight mix and 100 µL of sterile water. Stained slides were incubated in the dark at room temperature for 30 min following which the wells were then washed with sterile PBS and biofilm viewed using ×100 oil immersion on a Nikon ECLIPSE E400 (Nikon) microscope utilising a dual-band emission filter (450-490 nm/510-560 nm). Images were generated using NIS-Elements BR (Nikon) software version 3.22.09.

2.2.10 Preparation of *Clostridium difficile* strain 630 Spores

For efficacy testing of sporicidal GS preparations, *C. difficile* spores were prepared from *C. difficile* strain 630. BHI-S broth was inoculated with freshly grown *C. difficile* strain 630 and incubated at 37 °C for seven days in an anaerobic environment using a Whitley mg500 anaerobic workstation (DW Scientific, UK). Following the seven day incubation period cultures were transferred to 4 °C and incubated for a further 24 h (Sorg and Sonenshein, 2008). Aliquots (1 mL) were centrifuged at 10,000 rpm in an Eppendorf 5418R Centrifuge for 15 min, the supernatant discarded and spore pellets re-suspended in 1 mL of sterile water; this wash was repeated 10 times for each 1 mL aliquot as per Lawley *et al.* (2009). Spore suspensions were then heat treated to kill any remaining vegetative cells, by incubation at 60 °C in a Grant micro tube. Purification of spores was verified by plating onto both 0.1%

taurocholic acid-supplemented (Sigma-Aldrich, UK) and non-taurocholate supplemented BHI-S, and comparing colony counts after 24 h anaerobic incubation at 37 °C. Growth on taurocholate supplemented agar plates combined with lack of growth on non-taurocholate supplemented media indicated spore purity.

2.2.11 Assessment of GS48 and GS48-55 on Spores

To determine the efficacy of GS48 and GS48-55 against *C. difficile* strain 630 spores, the method of sporicidal suspension testing described by Vohra and Poxton (2011) was used. To a 1 mL aliquot of spore suspension, 0.1 mL of GS48, GS48-55 or sterile water (untreated control) was added. Tubes were briefly vortexed and then incubated at room temperature for varying time periods (0 h, 1 h, 4 h, 8 h, 12 h and 24 h). At each time point, samples were centrifuged at 13,000 rpm (using Eppendorf 5418R Centrifuge) for 5 min to recover spores, and the supernatant containing the disinfecting agent was removed. Harvested spore pellets were re-suspended in 1 mL sterile deionised water and 0.1 mL was then plated onto BHI-S agar supplemented with 0.1% (w/v) taurocholic acid to enhance germination (Burns *et al.*, 2010; Heeg *et al.*, 2012). Plates were incubated anaerobically at 37 °C and colony enumeration was performed every 24 h for a total of 5 days to allow complete germination as per the method of Heeg *et al.* (2012).

2.2.12 Statistical Analysis

For bactericidal testing, Log_{10} changes in viable bacterial numbers, compared to untreated controls was determined. The equation Log Reduction LR = Log_{10} ($N_{control}$) – Log_{10} ($N_{treated}$) was used where $N_{control}$ is total recovery of untreated bacteria and $N_{treated}$ is total recovery of

treated bacteria. Data was imported to Graphpad Prism 6.01 and charts constructed. Statistical analysis was completed using SPSS v22.

2.3 Results

2.3.1 Direct Bactericidal Assessment of GS5

We firstly wished to determine if GS5 was effective against bacteria in solution, prior to surface testing. We hypothesised that a solution of GS5 at working concentration would exhibit a bactericidal effect against a suspension of bacteria. The direct antibacterial effects of GS5 against *S. aureus* ATCC43300 was assessed using a suspension assay. *S. aureus* ATCC43300 was challenged with increasing concentrations of GS5 to quantify bactericidal activity. GS5 exhibited bactericidal actions at all concentrations after 5 min contact time (0.25% = 4.96 Log_{10} reduction; 0.5% = 5.6 Log_{10} reduction; 1% = 6 Log_{10} reduction (complete kill) (Figure 2.3). Subsequent testing was completed at 5% as per manufacturer's instructions.

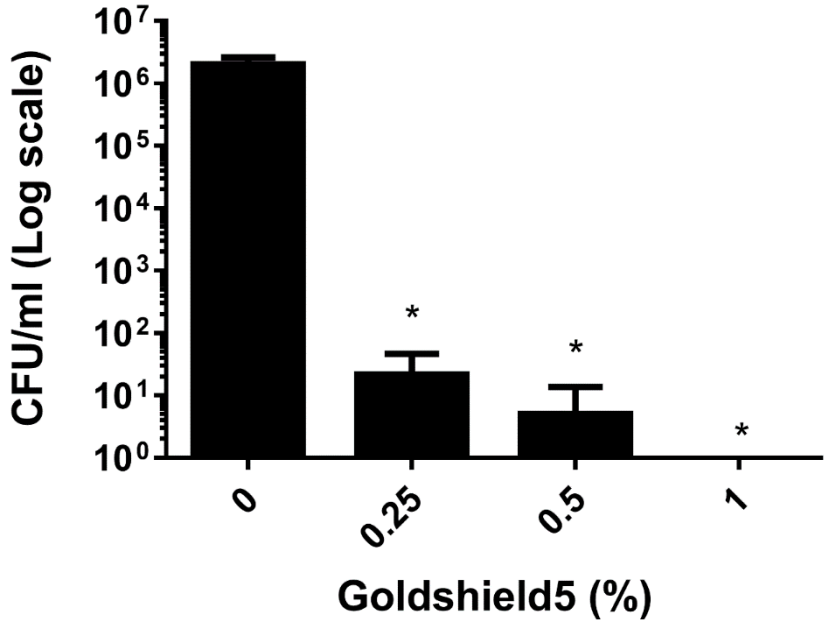

Figure 2.3 – *Staphylococcus aureus* ATCC43300 survival following suspension test using GS5. ~2×10^6 CFU/mL of *S. aureus* ATCC43300 was challenged with increasing concentrations of GS5. Data represents mean +/- SD of three independent experiments. Statistical analysis by independent T-tests versus Untreated (0%) controls (*=$p<0.05$).

2.3.2 Growth Calibration

For each antimicrobial testing protocol it was important to accurately and precisely apply a pre-defined number of bacterial to test surfaces. Therefore, a growth calibration to determine relationship between D_{600nm} to CFU/mL was completed. Figure 2.4 and Figure 2.5 show the growth calibration curves for Gram negative and Gram positive bacteria respectively; results are summarised in Table 2.2. This calibration was verified for each antimicrobial test by determining the CFU/mL of the bacterial suspension used in these tests, i.e. bacterial suspension applied to untreated surfaces and treated surfaces.

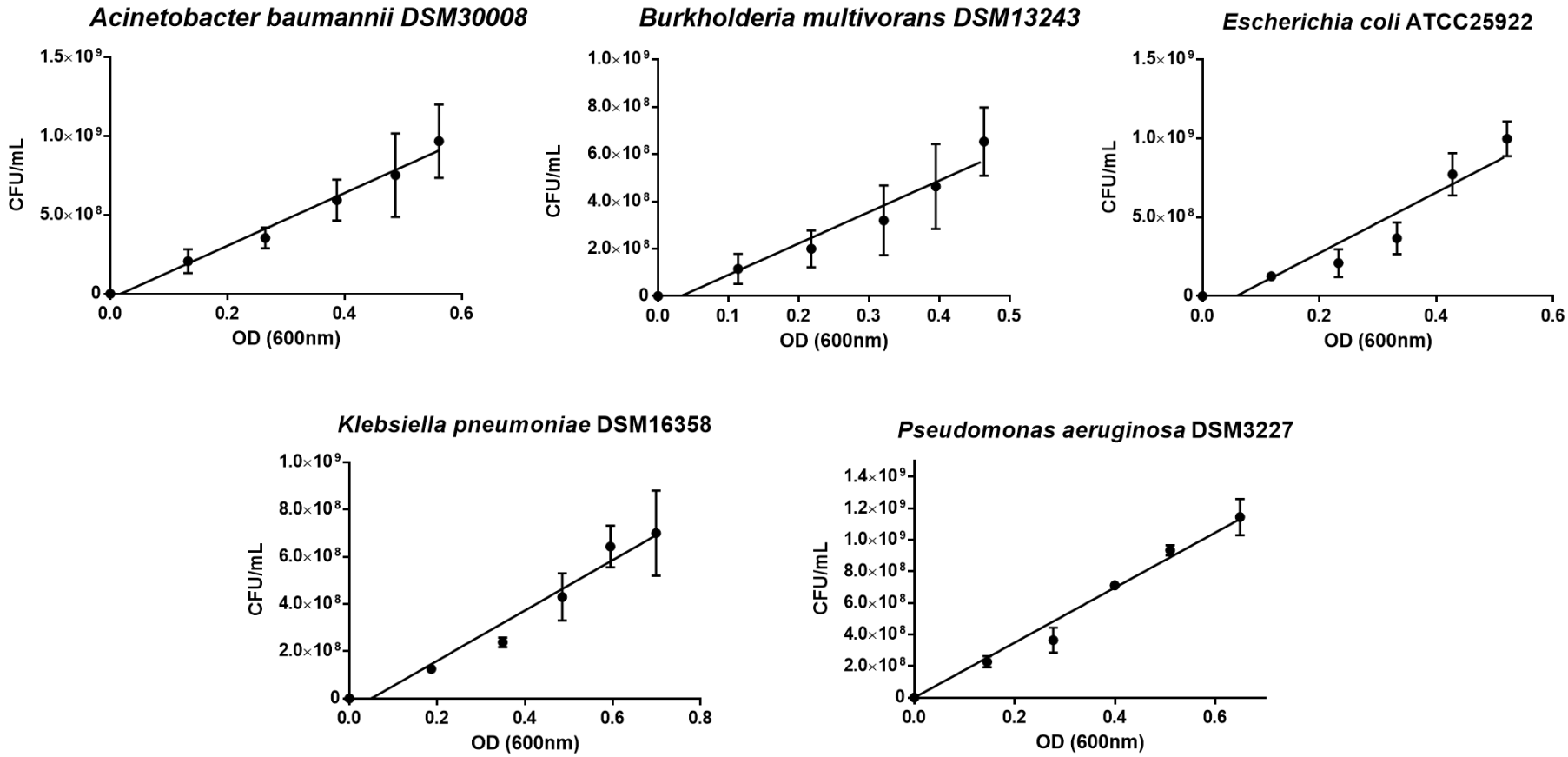

Figure 2.4 – Growth calibration curves for Gram negative model HAI bacteria. Graphs represent D_{600nm} to CFU/mL relationship for each bacterium. Data represents mean +/- SD of three independent experiments.

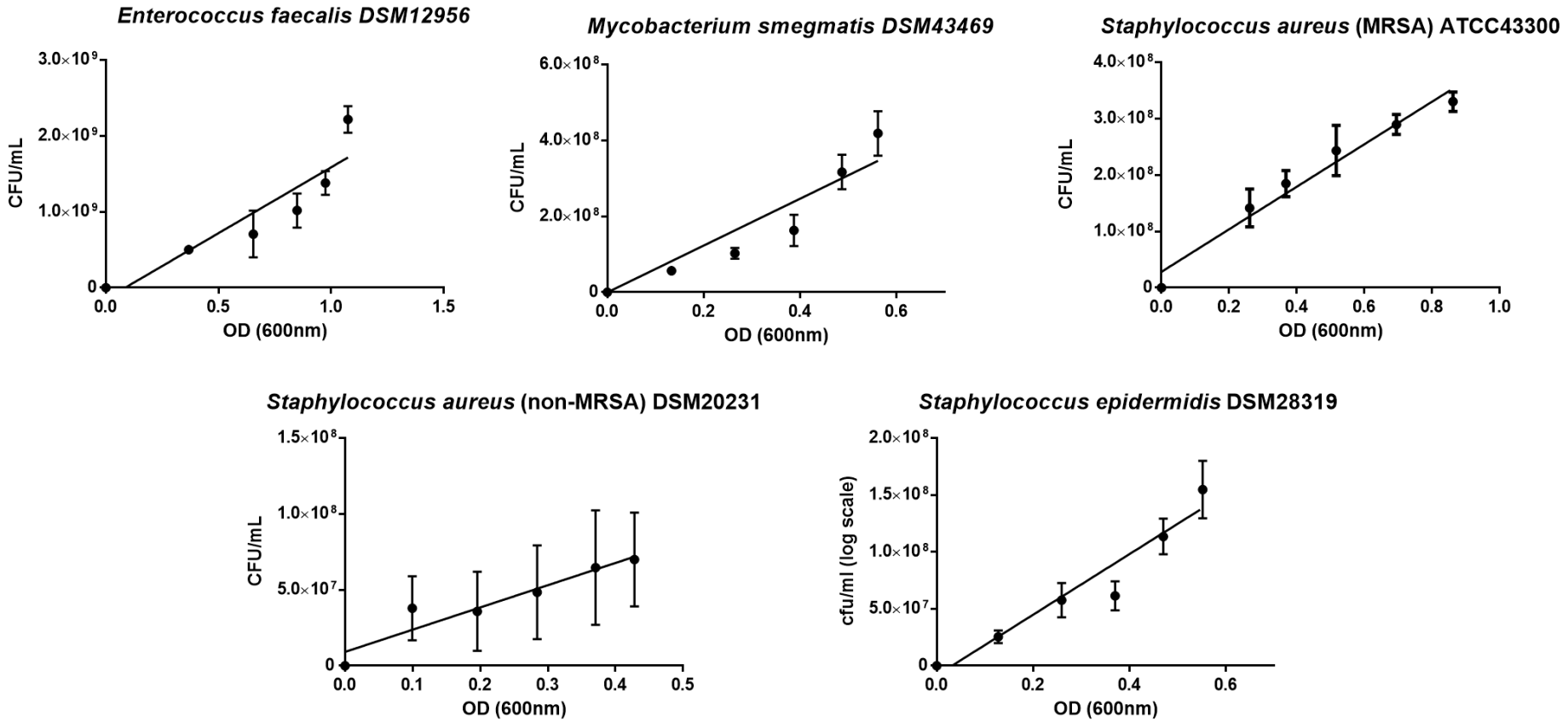

Figure 2.5 – Growth calibration curves for Gram positive model HAI bacteria. Graphs represent D_{600nm} to CFU/mL relationship for each bacterium. Data represents mean +/- SD of three independent experiments.

Table 2.2 – Growth calibration to determine optical density (at 600nm) to CFU/mL relationship for model healthcare acquired infection bacteria.

Bacteria	**y intercept**	$D_{600nm} = 2 \times 10^8$ **CFU/mL**
Acinetobacter baumannii DSM30008	y = 1.674e+009*x - 2.968e+007	0.12
Burkholderia multivorans DSM13243	y = 1.328e+009*x - 4.340e+007	0.16
Enterococcus faecalis DSM12956	y = 1.735e+009*x - 1.480e+008	0.13
Escherichia coli ATCC25922	y = 1.918e+009*x - 1.102e+008	0.11
Klebsiella pneumoniae DSM16358	y = 1.066e+009*x - 5.365e+007	0.20
Mycobacterium smegmatis DSM43469	y = 6.166e+008*x - 0.0	0.32
Pseudomonas aeruginosa DSM3227	y = 1.745e+009*x - 0.0	0.11
Staphylococcus aureus (MRSA) ATCC43300	y = 3.772e+008*x + 2.830e+007	0.49
Staphylococcus aureus (non-MRSA) DSM20231	y = 1.464e+008*x + 9.160e+006	1.29
Staphylococcus epidermidis DSM28319	y = 2.662e+008*x - 8.339e+006	0.78

2.3.3 Residual Activity of Surface Disinfectants

GS5 is reported to form covalent bonds with surfaces, thereby leaving a nanoscale antimicrobial coating which kills microbes that encounter that surface. This, it is claimed, makes GS5 a more effective product due to its residual antimicrobial activity compared to other disinfectants. We designed an experiment to test this hypothesis by determining the residual antimicrobial effect of GS5, Actichlor and Distel. The bactericidal activity of the three surface disinfectant agents was tested against *S. aureus* ATCC43300 on 316l Steel (Aalco, UK) and residual activity was assessed over 15 days at 3 day intervals. All three products exhibit bactericidal activity on day 0 (Actichlor = 3.75 Log_{10} reduction; Distel = 0.54 Log_{10}reduction; GS5 = 1.16 Log_{10} reduction). Following subsequent re-challenge of treated surfaces with *S. aureus* ATCC43300 only GS5 showed significant residual bactericidal activity, which was evident for a total of 6 days (Day 3 GS5 =0.53 Log_{10} reduction; Day 6 GS5 = 0.26 Log_{10} reduction; Figure 2.6). For subsequent testing of the GS5 product, the maximum effect time point (day 0) was used.

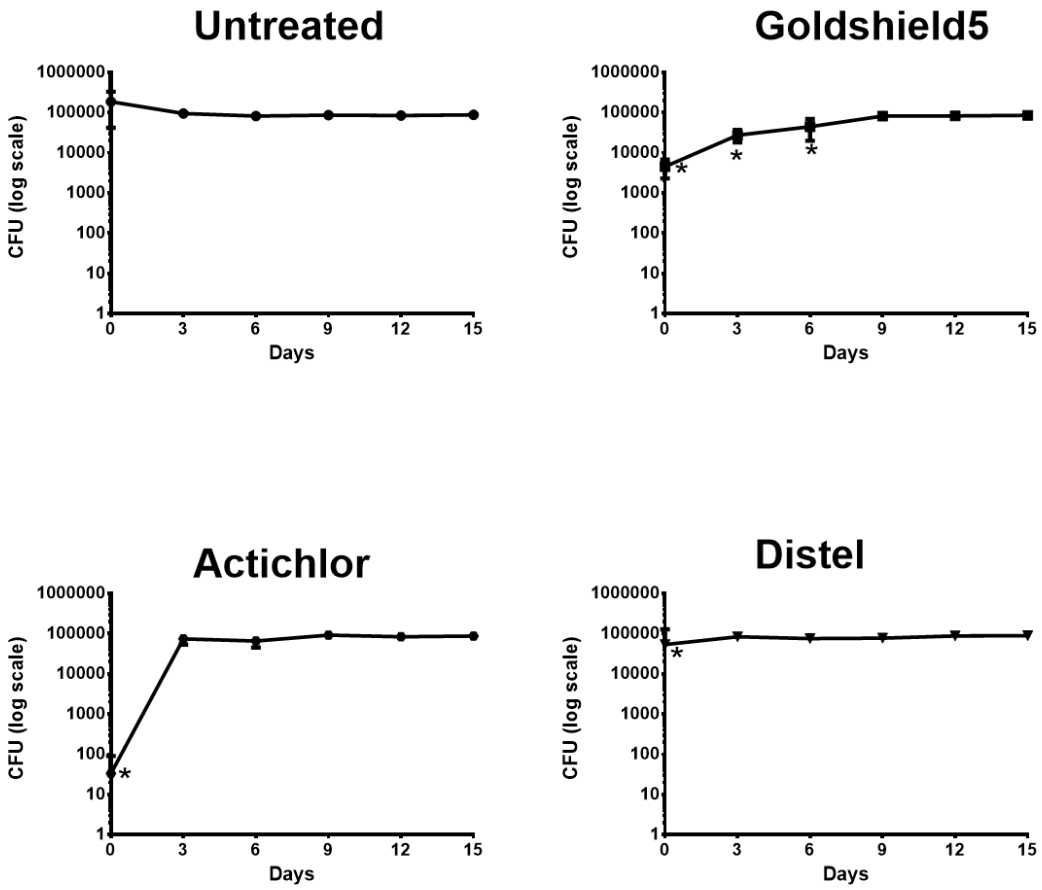

Figure 2.6 – Comparison of residual antimicrobial effects of GS5, Actichlor and Distel on steel surface loaded with *Staphylococcus aureus* ATCC43300. GS5 exhibited prolonged antibacterial activity (6 days) whereas Actichlor and Distel showed no antibacterial activity after day 0.

Results are representative of three independent experiments (n=3; mean+/- SD). Statistical analysis using One way ANOVA and Dunnett's T-test versus Untreated control (*=p<0.05, **=p<0.005, ***=p<0.001). ▪ = Goldshield; ● = Untreated control; ○ = Actichlor; □ = Distel.

2.3.4 GS5 Bactericidal Surface Testing

Baxa *et al.* (2011) reported that GS5 exhibited variable effects against different bacterial species. We therefore tested GS5 against a range of healthcare acquired infection microorganisms on 316l Steel or Formica to determine bactericidal effect. As hypothesised, GS5 treated surfaces did exhibit a bactericidal effect against all ten tested microorganisms, and this effect was observed on both Formica and steel. The largest bactericidal effect was observed with *Staphylococci* strains where a >1 Log_{10} reduction was observed on 316l Steel (*S. aureus* ATCC43300 = 1.21 Log_{10} reduction; *S. epidermidis* DSM28319 = 1.06 Log_{10}reduction) (Table 2.3). On Formica, however, the GS5 product exhibited a lower bactericidal effect (<0.5 = Log_{10}reduction) against both *Staphylococcus* organisms. The average Log_{10} reduction on steel surfaces for all bacteria tested was 0.6 Log_{10}reduction, whereas the average reduction on Formica was 0.45 Log_{10} reduction.

Table 2.3 – Log_{10} reductions obtained on GS5 treated surfaces challenged with a variety of microbes.

Organism	Surface	Log_{10} Untreated ± SD	Log_{10} Treated ± SD	Log_{10} change
Acinetobacter baumannii DSM30008	Steel	4.82 ±0.36	4.49 ±0.62	**0.33***
	Formica	4.23 ±0.04	3.67 ±0.29	**0.56***
Burkholderia multivorans DSM13243	Steel	3.89 ±0.14	3.61 ±0.17	**0.28****
	Formica	3.93 ±0.05	3.40 ±0.24	**0.53***
Enterococcus faecalis DSM12956	Steel	5.26 ±0.3	4.79 ±0.08	**0.47**
	Formica	5.15 ±0.13	4.86 ±0.03	**0.29**
Escherichia coli ATCC25922	Steel	5.57±0.28	5.32 ±0.33	**0.25**
	Formica	5.54 ±0.09	5.23 ±0.02	**0.31****
Klebsiella pneumoniae DSM16358	Steel	4.28±0.27	3.53 ±0.33	**0.75***
	Formica	3.93 ±0.05	3.40 ±0.24	**0.53****
Mycobacterium smegmatis DSM43469	Steel	4.15 ±0.22	3.46 ±0.45	**0.69***
	Formica	5.83 ±0.43	5.15 ±0.44	**0.68**
Pseudomonas aeruginosa DSM3227	Steel	5.09±0.04	4.66±0.29	**0.43***
	Formica	5.15±0.1	4.63±0.12	**0.52***
Staphylococcus aureus (MRSA) ATCC43300	Steel	4.20 ±0.13	2.96 ±0.58	**1.24****
	Formica	5.00 ±0.03	4.68 ±0.08	**0.32****
Staphylococcus aureus (non-MRSA) DSM20231	Steel	4.13±0.22	3.03±0.27	**1.1***
	Formica	5.02±0.23	3.94±0.35	**1.08**
Staphylococcus epidermidis DSM28319	Steel	3.95 ±0.04	2.88 ±0.05	**1.07***
	Formica	5.23 ±0.19	4.94 ±0.25	**0.29***

Results are representative of three independent experiments (n=3; mean+/- SD). *p* value calculated using T-Test (*=p<0.05, **=p<0.005, ***=p<0.001).

2.3.5 Effect of GS5 on Bacterial Biofilm Formation

Walker *et al.*, 2014 have demonstrated that biofilm contamination can contribute significantly to outbreaks of healthcare acquired infections. Given the efficacy of GS5 against a range of HAI microbes, we hypothesised that a GS5-treated surface would impede the development of bacterial biofilms. *P. aeruginosa* is a well characterised biofilm former (Webb *et al.*, 2003), and therefore we pre-treated plastic microtitre plate surfaces with GS5 and assessed the development of *P. aeruginosa* DSM3227 biofilms. The crystal violet staining method provides a quantitative measure of biofilm development/biomass and somewhat unexpectedly our data revealed that GS5 did not appear to inhibit the development of *P. aeruginosa* DSM3227 biofilm in plastic microtiter plates (Figure 2.7). Having observed that

P. aeruginosa DSM3227 biofilm development was apparently unaffected, we assessed bacterial viability within the biofilms using the well-established BacLight staining method. This analysis suggested that a proportion of the bacterial cells were damaged or rendered non-viable when grown on GS5 treated surfaces, but that, critically, a sufficient number of viable/undamaged cells remained (Figure 2.8) which, we hypothesise are responsible for subsequent biofilm development.

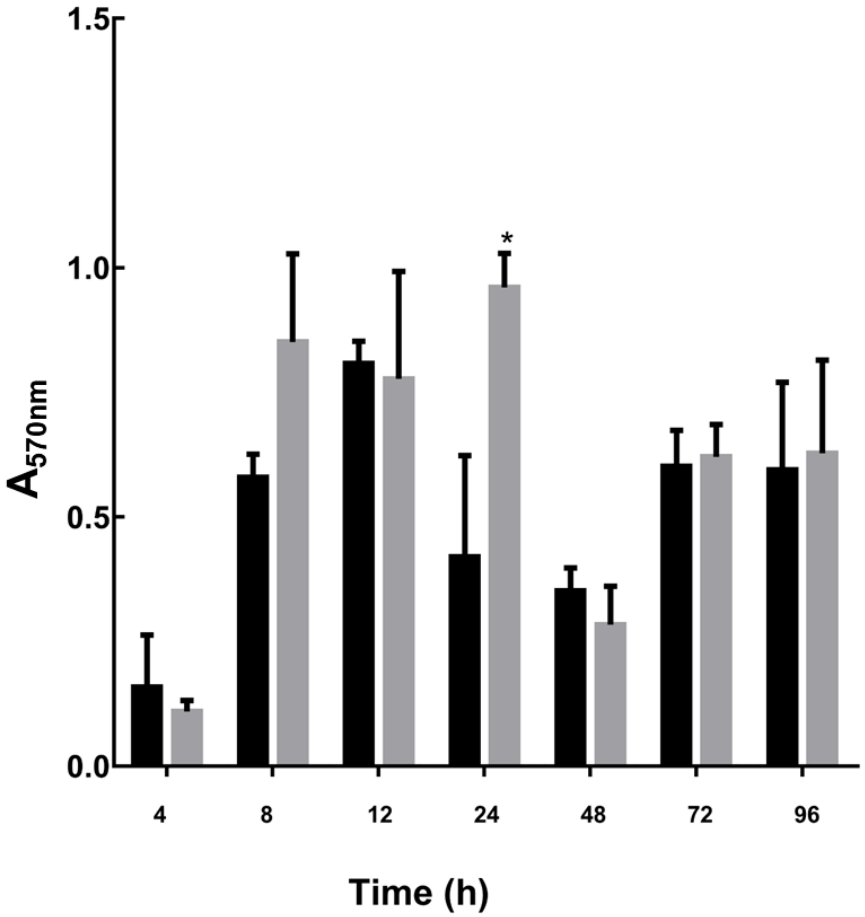

Figure 2.7 – Biofilm development following pre-treatment with GS5. *Pseudomonas aeruginosa* DSM3227 biofilm biomass was assessed by crystal violet staining at various time points and data presented represents mean +/- SD of three independent experiments. Statistical analysis by independent T-tests versus Untreated controls (*=p<0.05). Grey columns representative of pre-treated samples; black bars representative of untreated controls.

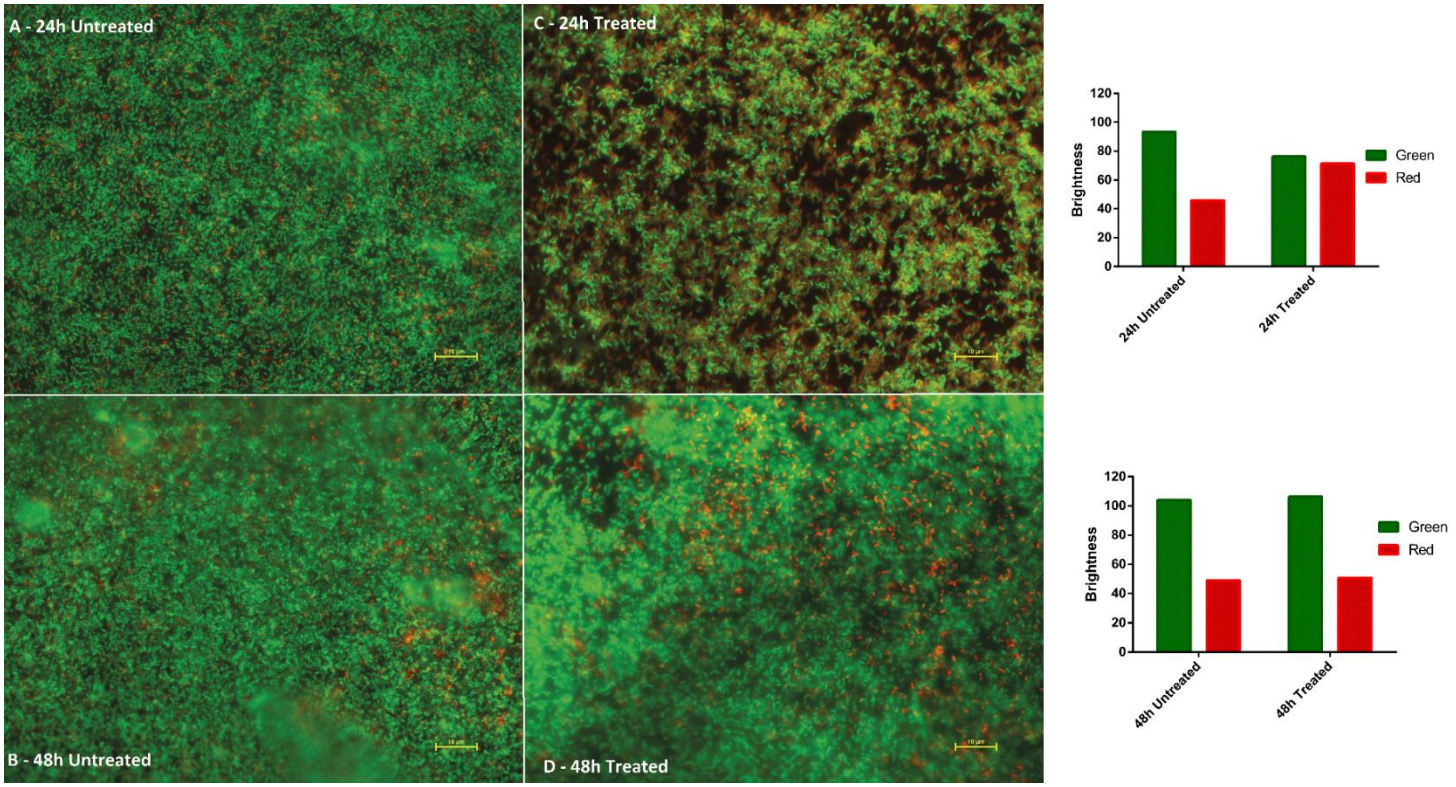

Figure 2.8 – BacLight staining of *P. aeruginosa* DSM3227 biofilm at 24 h and 48 h following pre-treatment with GS5. Live cells appear green and dead/damaged cells appear red. Images A and B show development of extensive biofilm on untreated surfaces. Image C shows biofilm development on GS5 treated surface with a greater proportion of dead/damaged cells. Image D shows GS5 treated surface biofilm at 48h: biofilm development and cell viability is similar to the untreated control. Images were obtained ×100 magnification (oil immersion) on a Nikon ECLIPSE E400 (Nikon) microscope utilising a dual-band emission filter (450-490nm/510-560nm) and NIS-Elements BR (Nikon) software; composite (red/green) images generated using ImageJ software. Scale bar = 10 µm.

2.3.6 Sporicidal activity of GS48 and GS48-55

GS48 and GS48-55 sporicidal products were tested for sporicidal activity against *C. difficile* strain 630 spores (Figure 2.9). Both agents exhibited sporicidal activity and we observed a marked reduction (>4 Log_{10}) in viable *C. difficile* strain 630 spores. A time-dependant sporicidal effect was observed with both GS48 and GS48-55 products with 4-5 Log_{10} reduction of spores following 8 h exposure to the wet GS product. An exposure time of 8-12 h was necessary for complete spore kill. We noted that GS48-55 exhibited a quicker kill-rate than the original GS48 product.

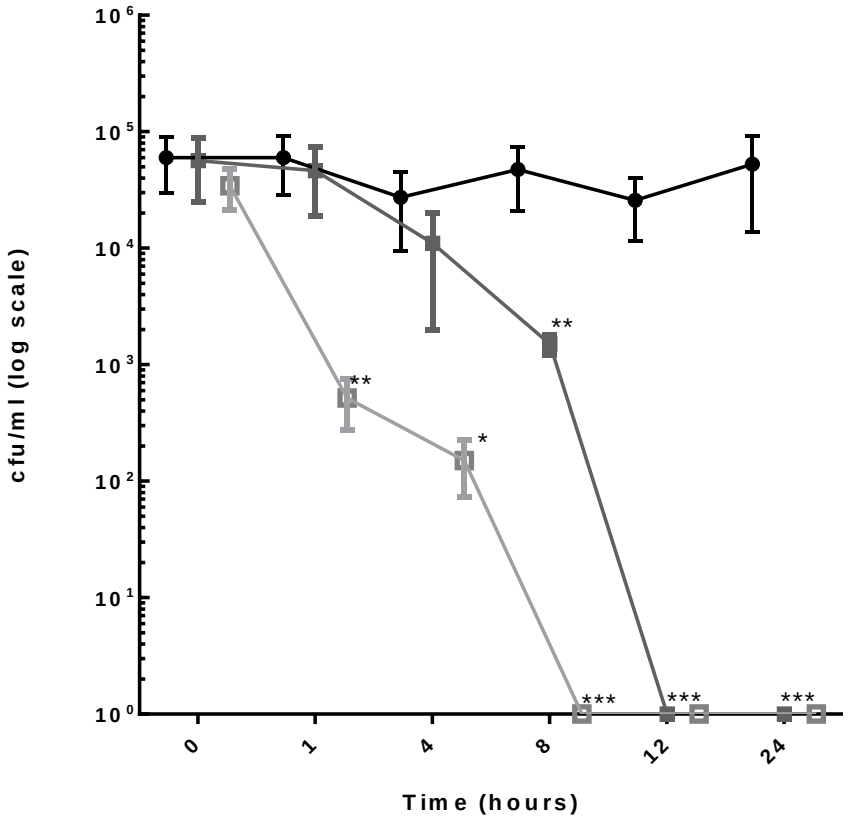

Figure 2.9 – Effect GS48 and GS48-55 sporicidal agents against *Clostridium difficile* strain 630 spores. Data presented is mean+/- SD of three independent experiments. Statistical analysis by One way ANOVA and Dunnet T-tests versus Untreated (*=p<0.05, **=p<0.005, ***=p<0.001). ● = Untreated control; ▪ = GS48; □ = GS48-55.

2.4 Conclusion

Only a single published report exists (Baxa *et al.,* 2011) which details the effects of GS5 used as a surface biocide. GS5 is reported to exert its antimicrobial effect via bonding of the silane end of the molecule to surfaces, following which microbes are drawn onto the hydrocarbon chain. The resultant puncturing of cell membranes and denaturation of proteins is proposed as the cause of cell death (Baxa *et al.*, 2011; Perez *et al.*, 2015). As a covalent bond is formed with the surface it is hypothesised that this mode of action is prolonged creating a 'bactericidal surface'.

2.4.1 GS5 as a Long Lasting Surface Disinfectant

The residual activity of GS5 exhibited bactericidal activity for 6 days (0.26 Log_{10} reduction) whereas the other surface disinfectants tested showed no activity beyond day 0 (Figure 2.6). In comparison with previous residual testing of the GS5 product (Bax*a et al*, 2011), which was completed on fabric swatches rather than on hard surfaces, we observed that residual antimicrobial activity of GS5 was lower (6 days rather than 14 days). However, the residual antibacterial effect decreased over time to a <1 Log_{10} reduction in bacterial numbers, suggesting that GS5 would need regular reapplication and would not be sufficient as a surface disinfectant alone.

GS5 treated surfaces exhibited bactericidal activity which varied in effectiveness between surface type and bacterial species. Thus, bacterial species challenged, in addition to surface type/properties, appears to have a significant influence on the performance of the GS5 product. Surface hydrophobicity, charge and roughness have all been reported as important

with respect to performance of biocides (Beggs *et al.*, 2015). Indeed, variations in the response of bacterial species to disinfectants is evident in the literature with disparate Log_{10} reductions and widely varying minimum inhibitory concentrations (MICs); biocidal resistance is also evident (Baxa *et al.*, 2011; Otter *et al.*, 2015). GS5 is said to not induce resistance in microorganisms as a result of its physical mode of action, reported as membrane disruption and protein denaturation. We noted differences between the results of our current work and data reported by Baxa *et al.* (2011) who also tested *S. aureus*, *E. coli* and *P. aeruginosa* on steel and Formica. The work of Baxa *et al.* (2011) suggested that GS5 had greater efficacy against *E. coli* and *P. aeruginosa*, however this observation could be a result of differing surface properties across different types of Steel and Formica used. However, like Baxa *et al.* (2011), we have shown that the performance of GS5 against different bacterial species varies considerably, which indicates that the specific type of microbial contaminant will be of greater influence on the effectiveness of GS5, than the actual surface on which it is used.

Methods to assess GS technology coating capabilities were used, such as contact surface angle measurements, bromophenol blue indicator and scanning electron microscopy however results proved inconclusive. However the antimicrobial activity of coated surfaces compared to control surfaces strongly indicates successful GS coating.

2.4.2 GS5 Use for Prevention of Biofilm Formation

Experiments in which plastic surfaces were pre-treated for 10 min with GS5 showed that there was no significant inhibitory effect against *P. aeruginosa* biofilm formation. It is well documented that biofilms exhibit increased resistance to antimicrobials and disinfectants, mainly due to the inability of these molecules to penetrate the biofilm (Otter *et al.*, 2015;

Devlin-Mullin *et al.*, 2017). Given that the GS5-treated plate surfaces would be expected to possess antimicrobial activity, we then considered the viability of cells within developing biofilms. Using BacLight, we observed an initial apparent bactericidal effect on *P. aeruginosa* DSM3227 cells as evidenced by a reduction in biofilm coverage and increased numbers of red stained, damaged, cells at 24 h. However, this did not result in reduced biofilm formation as measured by crystal violet staining, and 48 h samples showed a well-developed biofilm containing viable cells, similar to that observed in the untreated control. It is likely, therefore, that residual viable cells maintain the ability to form biofilm and we hypothesise that the cells that are initially damaged by GS5 could actually promote biofilm formation: it has been suggested that dead bacterial cell constituents could comprise a key component of the biofilm or indeed even enhance adhesion and stability of cells, thereby allowing biofilm development (Bayles, 2007). The data assessing the quantitative and qualitative effects of GS5 on *P. aeruginosa* biofilm suggest that GS5 treatment will not significantly inhibit biofilm formation.

2.4.3 GS48 and GS48-55 Sporicidal Activity

Both GS48 and GS48-55 products exhibited considerable sporicidal activity when spores were suspended in the product, with GS48-55 having a quicker kill rate. Given that 3% hydrogen peroxide has been shown to be sporicidal against *C. difficile* spores (Lawley *et al*., 2010) this is unsurprising as the GS48-55 contains a higher level of hydrogen peroxide than GS48. A number of papers have reported use of Hydrogen peroxide vapour (HPV) systems for de-contamination of hospital rooms with specific interest in the efficacy of these against *C. difficile* spores (Boyce *et al.*, 2008; Shapey *et al.*, 2008; Passaretti *et al.*, 2013). While many of these reports have shown very effective reduction of both contamination and infection rates, there is however evidence of rapid re-contamination of environments post de-

contamination (Hardy *et al.*, 2007; Aldeyab *et al.*, 2009). Whilst the GS48 and GS48-55 sporicidal agents are, in our hands, highly sporicidal *in vitro*, the efficacy of these in a real clinical setting has yet to be demonstrated.

2.4.4 GS Technology use as a Hospital Disinfectant

Current NHS Infection control practices require that when choosing disinfectants, a 4–5 Log_{10} reduction is required in viable vegetative bacterial cells within a contact/drying time of 10 min, in addition to a spore reduction of 3 Log_{10} within the same period. When tested directly on a suspension of bacterial cells, GS5 achieved a more than 4 Log_{10} reduction with a 5 min contact time however the residual surface active antimicrobial activity of GS5 was much less, at approximately 1 Log_{10} reduction in bacterial numbers. The surface protective effect of GS5 remained for a further 3–6 days without reapplication of the product, however we noted a diminution of the measured Log_{10} reductions over time to a level which was much lower than that required for use in infection control. Bacteria can form biofilm on surfaces allowing prolonged survival and increased resistance to biocides. Considering the GS5 mode of action we hypothesised a regime where GS5 could be utilised to prevent biofilm formation on surfaces subsequently reducing risk of infection. However GS5 has been shown to possess limited anti-biofilm properties as biofilm production is not impeded on GS5 coated surfaces.

Within the NHS, certain disinfectants (for example, DifficilS) routinely achieve 4 Log_{10} reductions in both vegetative cell and spore numbers within 3–5 min however control of infection is only achievable in practice by using these products in intensive cleaning up to twice daily in a rolling programme of disinfection. Thus, on the basis of the data generated

in this work, it appears unlikely, despite modest reductions in bacterial cell viability and evidence for a short lived residual effect, that GS5 would replace current infection control products such as DifficilS or Actichlor in reducing the transmission of HAI pathogens within hospitals and care settings.

Chapter 3

Assessment of the reservoir potential of healthcare workers' uniforms as a source of antibiotic resistant pathogenic bacteria

`3.1 Introduction

3.1.1 Prevalence of Healthcare Acquired Infections in Healthcare Settings

HAIs are infections that occur in healthcare settings at least two days after admittance to hospital (Cardoso *et al.*, 2014). It is estimated that HAIs are responsible for 4.5 million infections per year in the EU (European Centre for Disease Prevention and Control, 2018). HAIs occur in healthcare settings for a variety of reasons: there are multiple factors that are unique to healthcare settings (for example, hospitals) which encourage the emergence, prevalence and spread of HAIs. One such factor is increased antibiotic resistance, as over-use, mis-use and over-dependence on antibiotics in hospitals has resulted in the emergence of highly virulent, difficult to treat, multi-drug resistant organisms and their persistence in hospitals (Cosgrove, 2006). HAIs are predominantly opportunistic pathogens therefore the increased vulnerability of patients in hospitals is a contributing factor for increased prevalence and spread of HAIs. However, it is not just simply these factors individually that can cause a problem but a combination, that increases prevalence of HAIs. For example use of antibiotics can increase vulnerability of patients to opportunistic organisms. Use of antibiotics can result in suppression of the normal microbiota reducing protectiveness against colonisation by opportunistic pathogens, and this is especially true when broad spectrum antibiotics are used. As these increase the risk of patient's developing *C. difficile* infection (CDI) – a common HAI (Bartlett, 2002; Jernberg *et al.*, 2010, Deshpande *et al.*, 2017).

3.1.2 Contamination of Healthcare Settings

As discussed in chapter 1, contamination of the healthcare setting is a source of spread of bacteria onto patients (Mitchell *et al.*, 2013; Dancer, 2014, 2016; Lee *et al.*, 2018). The most

problematic areas tend to be so called “high-touch” points with examples including bed rails, door handles, table top surfaces, bed clothing (linen and mattress), staff, and staff uniforms. As a consequence, the CDC guidelines specifically stipulate that *“close attention be paid to cleaning and disinfecting high touch surfaces in patient care areas”* (Carling *et al.*, 2008). When these inanimate surfaces become contaminated with infectious agents they then act as a source for spread of bacteria to patients both directly and indirectly. Bacteria can be contacted by patients (direct transmission) or staff who thus indirectly spread bacteria to patients (via hands, uniforms or both) – ultimately direct and indirect spread could potentially result in infection of patients resulting in increased infection rates and associated costs. The transmission routes are summarised in Figure 3.1.

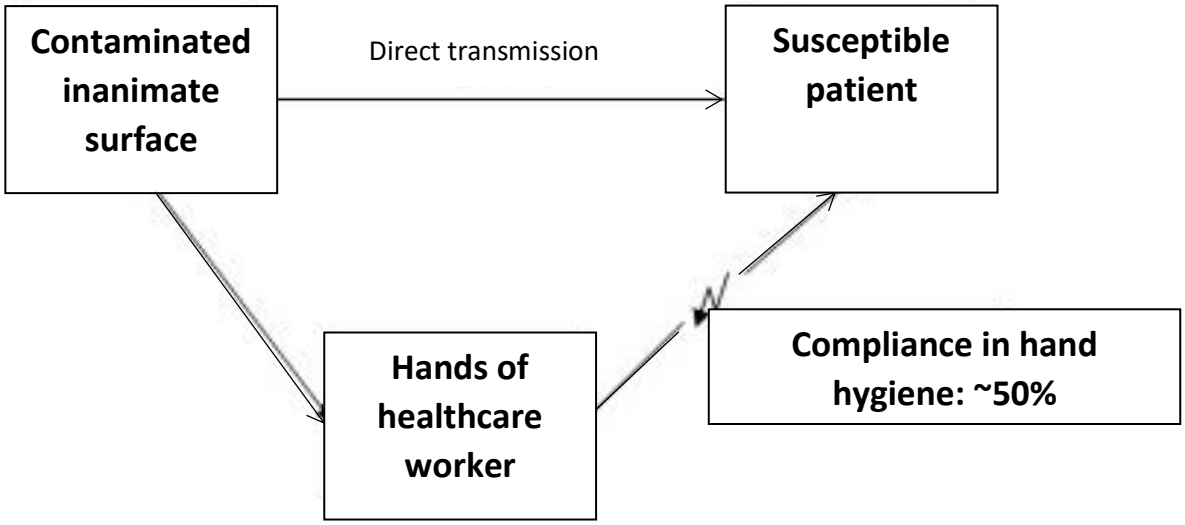

Figure 3.1 – Direct and indirect transmission routes for dissemination of Healthcare acquired infections from contaminated surfaces in healthcare settings onto patients (Kramer *et al.*, 2006).

3.1.3 Contamination of Healthcare Workers Uniforms

Healthcare workers' uniforms can act as vectors in the indirect transmission of bacteria to patients (Figure 3.1) and in fact contamination of workers' hands often results in contamination of uniforms and *vice versa* (Munoz-Price *et al.*, 2012). It is well documented that healthcare workers' uniforms are contaminated with bacteria which could potentially act as an infectious agent toward patients (Abu Radwan and Ahmad, 2017; Gaspard *et al.*, 2009).

For example, in an investigation to identify types of microorganisms present on a variety of healthcare professionals' uniforms, 305 samples were tested and the investigators identified 24 types of microorganisms including *S. aureus*, *S. epidermidis*, *Bacillus* spp., and *Acinetobacter* spp., amongst others (Abu Radwan and Ahmad, 2017). In a study assessing MRSA contamination of uniform pockets in a long-term care facility, high levels of MRSA contamination were observed. In standard non-controlled (no changes to normal practice) uniforms, 60% of pockets were contaminated with MRSA. Subsequently, a range of test groups were set up, each with varying instructions for prevention of contamination (examples of instructions included wearing of aprons to zero use of pockets). The authors found that only the groups with the strictest instructions – meaning disposable aprons were worn, hand hygiene instructions were followed and zero pocket contents – and those that demonstrated complete compliance with these instructions had 0% contamination (Gaspard *et al.*, 2009). This investigation elegantly highlighted how easily the hospital environment results in uniforms becoming contaminated.

3.1.4 Use of Antimicrobial Healthcare Workers Uniforms

Due to the high levels of uniform contamination a number of interventions have been tested with the aim of reducing/removing this bioburden. Examples of such interventions include the use of antimicrobial fabrics, or specialised laundry conditions. Researchers at Denver Health Hospital, Colorado, USA assessed 105 workers' uniforms with the sample groups being made up of 5 types of hospital workers and 3 different fabric types (standard uniforms and type A and type B 'antimicrobial' uniforms). These were assessed for bioburden after an 8 h working day. All the uniforms assessed were contaminated with bacteria and no significant differences were seen between control groups and furthermore antimicrobial fabric groups (Burden *et al.*, 2013). Another study conducted at Antrim Area Hospital assessed the effects of antimicrobial impregnated fabrics and in this work all uniforms tested demonstrated bacterial contamination. Again there was no difference in contamination levels reported between standard/control uniforms and "antimicrobial" impregnated uniforms. (Johnston, 2012).

3.1.5 Spread of Antibiotic Resistance by Contamination of Healthcare Workers Uniforms

Contamination of healthcare workers' uniforms poses a significant risk to patients in healthcare settings as this contamination has been shown to affect infection rates (Gaspard *et al.*, 2009; Sanon, 2012; Burden *et al.*, 2013; Abu Radwan and Ahmad, 2017). However, as contamination of uniforms contributes to the spread of HAIs (Johnston, 2012; Deshpande *et al.*, 2017) this contamination subsequently promotes the survival and resistance of these microorganisms within the healthcare environment (Kramer *et al.*, 2006b; Dancer, 2014). As these microorganisms continue to persist in the healthcare environment there is the

potential for development of increased numbers of antibiotic resistant strains (O'Neill, 2016). Such microorganisms could already be highly pathogenic multi-drug/antibiotic resistant strains, or they could adapt – for example by acquiring additional genetic material – to become multi-drug resistant whilst persisting in the hospital environment (Weinstein, 2001). In conclusion the survival/spread of HAIs via healthcare workers uniforms is a concern for two major reasons, [1] increased infection risk and, [2] increased antibiotic resistance.

3.1.6 Aims and Hypothesis

The main aim of this chapter was to robustly assess bacterial contamination of healthcare workers' uniforms at a local hospital. Thus, the first aim of this work was to develop a highly sensitive detection, recovery and enumeration protocol for recovery of bacteria from fabric surfaces. This method was then implemented to enumerate *S. aureus* and *Enterococcus* spp. contamination on pre-shift and post-shift healthcare workers' uniforms at Antrim Area Hospital, Northern Health and Social Care Trust. A biobank was created from the bacteria isolated from uniforms and these bacteria were assessed for antibiotic resistance/susceptibility profiles against commonly used antibiotics. It was hypothesised that pre-shift healthcare workers uniforms would have little or no *S. aureus* or *Enterococcus* spp. contamination, and that both *S. aureus* and *Enterococcus* spp. contamination levels would be increased significantly on post-shift healthcare workers' uniforms. It was also hypothesised that multi-drug resistant isolates would be identified in antibiotic susceptibility testing.

3.2 Materials and Methods

3.2.1 Study Overview

A pilot study was conducted at Antrim Area Hospital, Northern Health and Social Care Trust (NHSCT). The aim of the study was to determine *S. aureus* and *Enterococcus* spp. contamination levels on pre-shift and post-shift healthcare workers uniforms in a comparative assessment. Post-shift uniform contamination could be indicative of environmental contamination burdening healthcare workers uniforms. In addition we assessed was the antibiotic sensitivity profiles of randomly selected *S. aureus* and *Enterococcus* spp. isolated from post-shift uniforms.

A total of 100 pre-shift and 100 post-shift uniforms were assessed. The domestic services team at Antrim Area Hospital provides freshly laundered uniforms for staff (pre-shift) and collects uniforms to be laundered (post shift). Uniforms were sampled for microbial contamination at the pocket, abdominal area and neck equalling a total of 600 samples.

This work was funded by an NHS discretionary award secured by Jason Murray (Ulster University), Dr Nigel Ternan (Ulster University) and Prof Michael Scott (NHSCT). No ethical approval was required as no human or animal participants were involved.

3.2.2 Chemicals, glassware and media

All glassware was cleaned/sterilised by soaking overnight in 1% Virkon (Antec, UK) and steam sterilised in an autoclave prior to use. All culture media was prepared as per the manufacturer's instructions. For agar growth of presumptive *S. aureus* CHROMagar™ Staph aureus [CSA hereinafter] (Bioconnections, UK) was used. CSA was prepared by suspending in deionised water (82.5 g/L), bringing to boil on a Bibby HB502 hot plate (Bibby Scientific, UK) with stirring followed by dispensing into 9 cm petri dishes (SLS, UK). Slanetz and Bartley agar was prepared by suspending in deionised water (42 g/L), bringing to boil and dispensing into 9 cm petri dishes. All Slanetz and Bartley plates were incubated at 45°C with colonies enumerated daily for a total of 5 days. For Total Viable Counts (TVC) Tryptone Soya Broth/Agar (TSB/TSA) (Oxoid, UK). TSB was prepared by suspension in deionised water (30 g/L) and steam sterilised in an autoclave. TSA was prepared by suspension in deionised water (40 g/L) and steam sterilised in an autoclave prior to dispensing in 9 cm petri dishes. All TVC cultures were incubated at 37 °C. For broth growth of presumptive *S. aureus* TSB plus 7.5% (w/v) sodium chloride (Sigma, UK) was used (Goodwin and Pobuda, 2011). TSB plus 7.5% sodium chloride was prepared by suspension in deionised water (30 g/L), addition of 7.5% sodium chloride followed by steam sterilisation in an autoclave. Mueller-Hinton Broth/Agar (MHB/MHA) (Oxoid, UK) was used for biobank creation and growth in antibiotic susceptibility testing. MHB was prepared by suspension in deionised water (21 g/L) and steam sterilised in an autoclave. MHA was prepared by suspension in deionised water (38 g/L) and steam sterilised in an autoclave prior to dispensing in 9 cm petri dishes. All MHB and MHA were incubated at 37 °C. Phosphate Buffered Saline (Oxoid, UK) was prepared in deionised water and steam sterilised in an autoclave prior to use.

3.2.3 Optimisation of recovery and enumeration of bacteria

There is considerable dispute in the literature regarding the sensitivity and accuracy of techniques of direct plating of samples, or sample enrichment in the recovery, isolation and enumeration of microorganisms from inanimate surfaces/objects (Lesmana *et al.*, 1997; Mcallister *et al.*, 2011; Liss *et al.*, 2013). Moreover, direct plating versus pre-enrichment is particularly important in recovery from inanimate surfaces such as healthcare workers' uniforms (Landers, Hoet and Wittum, 2010) as it could be argued a pre-enrichement step would add bias (especially true for enumeration) and selection. If the researcher is wishing to detect a pathogen in a human or food sample, then it is suggested that a pre-enrichment step is more applicable in order to increase sensitivity of detection for low numbers (O'Brien *et al.*, 2005; Liss *et al.,* 2013). However if the user wishes to accurately enumerate microorganism(s) in a sample, then direct plating is more suitable (Landers *et al.*, 2010) as pre-enrichment will result in exaggerated counts which could be problematic in comparative studies. In this work, we decided to test both the direct plating approach and the pre-enrichment approach. One pre-shift uniform and one post-shift uniform were swabbed as described in the 'Uniform sampling' section. Briefly, from the 5 mL swab samples 0.1 mL was directly plated onto Tryptone Soya Agar (TSA) (Oxoid, UK) to determine total viable count (TVC) without pre-enrichment; and onto CSA to determine *S. aureus* count without pre-enrichment. All samples were plated out in duplicate, incubated at 37 °C overnight and colonies enumerated. In addition, 50 mL of Tryptone Soya Broth (TSB) (Oxoid, UK) was inoculated with a 50 μL (1% v/v) aliquot of the swab sample to determine TVC with enrichment. Similarly a 1% inoculation was completed into 50 mL TSB plus 7.5% sodium chloride (Goodwin and Pobuda, 2011) to determine *S. aureus* count with pre-enrichment. Both inoculations were incubated at 37 °C for 12 h followed by serial dilution in PBS and

plating onto TSA and CSA as described above. All plates were completed in duplicate, incubated at 37 °C overnight and colonies enumerated.

3.2.4 Uniform collection

100 pre-shift uniforms and 100 post-shift uniforms were directly collected from domestic services team at Antrim Area Hospital, NHSCT. Uniforms were collected at 9 am each morning, corresponding to the time at which daily collection of post-shift uniforms and daily allocation of pre-shift uniforms occurred (Table 3.4). Uniforms were individually packaged in UV-treated collection bags and transported to Ulster University, Coleraine (travel time = approximately 1 h).

3.2.5 Uniform sampling

Uniforms were sampled for recovery of microorganisms from their surface; individual samples were collected from the abdomen, neckline and pocket of the uniforms (200 uniforms × 3 sampling sites = 600 samples). Swabs were pre-moistened in sterile PBS (Oxoid, UK) followed by vortex recovery of bacteria as these conditions have been shown to result in higher sensitivity for bacterial recovery compared to other methods (Moore and Griffith, 2002; Hodges *et al.*, 2006, 2010; Landers *et al.,* 2010). Pre-moistened cotton swabs (Copan, UK) were used to swab each area by motioning the swab in a 15 up/down and 15 left/right motion over a 10 × 10 cm area of each sample site. Swabs were then transferred to 5 mL sterile PBS and subject to vortex for 1 min. Each sample was clearly labelled with uniform number and sample site. From each sample a serial dilution range was completed and each dilution spread plated in duplicate (0.1 mL spread plates) onto CSA and Slanetz and Bartley

agar. CSA plates were incubated at 37 °C and Slanetz and Bartley plates were incubated at 45 °C followed by colony counting for enumeration.

3.2.6 Bioburden analysis

Following enumeration, colony forming unit (CFU) values were obtained for each sample point *(CFU = colony count × 1/dilution factor × 1/volume plated in mL)*. Values were collated and an average count calculated for each sample area for pre-shift and post-shift uniforms. For comparison purposes, the log change in bacterial numbers between pre-shift and post-shift uniforms was determined *(Log Reduction LR = log_{10} ($N_{post\ shift}$)/log_{10} ($N_{pre\text{-}shift}$)).* Data was imported to Graphpad Prism 6.01 and charts constructed. Statistical analysis (Wilcoxon test) was completed using SPSS v22.

3.2.7 Creation of Biobank

As we wished to determine antibiotic susceptibility profiles, random selection of colonies deemed positive for *S. aureus* (pink/mauve on CSA) and colonies deemed positive for *Enterococcus* spp. (red/maroon on Slanetz and Bartley) were selected and isolated. Isolates were only selected from post-shift uniforms. These colonies were sub-cultured onto MHA and incubated overnight at 37 °C. Biomass from each isolate was transferred into cyrovials (Technical services consultants LTD, UK), given identification numbers and stored at -80 °C.

3.2.8 Antibiotic susceptibility testing using EUCAST guidelines

All biobank isolates were subject to antibiotic susceptibility testing using the disk diffusion method adhering to the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/). These guidelines provide information on procedures and analysis ensuring standardisation of antibiotic testing. Main points from the guidelines include use of Mueller Hinton as growth media, a growth density of 0.5 McFarland standard for all test cultures, antibiotic discs used at a maximum of four disks per plate and a series of resistant/sensitive breakpoints for a range of bacteria and specific antibiotic. To determine the antibiotic susceptibility profile each isolate was recovered from -80 °C by placing a bead into 5 mL of fresh MHB and incubating overnight at 37 °C. Simultaneously a duplicate bead was recovered onto fresh MHA and also incubated overnight at 37 °C; this plate was inspected following growth to ensure no contamination, i.e. single colony type on plate. From the inoculated MHB a 0.2 mL aliquot was transferred into 10 mL fresh MHB and incubated at 37 °C. Growth was regularly checked using a Pharmacia Biotech Novaspec II (Pharmacia LKB Biotechnology, Sweden) to measure attenuance at 600nm (D_{600nm}) using fresh MHB as a reference. When culture turbidity reached 0.5 McFarland standard (see McFarland standard section), 0.1 mL of culture was spread onto fresh MHA (4 plates per isolate). A total of 4 different antibiotic discs were then applied to the spread plates using an antibiotic disc dispenser (Oxoid, UK). Plates were incubated overnight (16 h) at 37 °C followed by measurement of zones of inhibition for each antibiotic (measuring the diameter of the circle surrounding each antibiotic using a ruler, measurements in mm). Each isolate was tested against each antibiotic in duplicate.

3.2.9 Antibiotic susceptibility testing analysis

Zone of inhibition averages were compared against a sensitive/resistant breakpoint defined by EUCAST (Table 3.1). Breakpoints are specific for bacterial genus/species against specific antibiotics. In some cases, however, no breakpoint is defined due to standardisation issues. If no breakpoint was provided the decision was made to use either the *S. aureus* breakpoint for *Enterococcus* spp. or vice versa; for example no breakpoint is provided for vancomycin against *S. aureus*; therefore the *Enterococcus* spp.-vancomycin breakpoint was used to determine antibiotic profile of *S. aureus* isolates. An antibiogram was then created for each of the sub-populations (*S. aureus* – abdomen, neck and pocket and, *Enterococcus* spp. – abdomen, neck and pocket). Bionumerics software (Applied Maths) was subsequently used to assess the diversity of antibiotic resistant profiles, (this analysis is reported in Chapter 4). Multi-drug resistance (MDR) was also determined. Collectively, EUCAST, CDC and ECDC define MDR as *"acquired non-susceptibility to at least one agent in three or more antimicrobial categories"* (Magiorakos *et al.*, 2012). The antibiotic profiles of *S. aureus* isolates were assessed to determine MDR or non-MDR classification. MDR profiles were not assessed for *Enterococcus* spp. isolates as official EUCAST sensitive/resistant breakpoints are only available for two of the antibiotics tested.

Table 3.1 – Antibiotics used in testing biobank isolates using European Union Committee for Antibiotic Susceptibility Testing (EUCAST).

Antibiotic Information			Breakpoints (mm)					
			Staphylococcus aureus			*Enterococcus* spp.		
	Code	Concentration (as defined by EUCAST)	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Cefoxitin	FOX	30 µg/mL	≥22		<22	≥22*		<22*
Vancomycin	VA	5 µg/mL	≥12*		<12*	≥12		<12
Penicillin G	P	10 units	≥26		<26	≥26*		<26*
Erythromycin	E	15 µg/mL	≥21	18-20.9	<18	≥21*	18.1-20.9*	<18*
Gentamicin	CN	10 µg/mL	≥18		<18	≥18*		<18*
Fusidic acid	FD	10 µg/mL	≥24		<24	≥24*		<24*
Clindamycin	DA	2 µg/mL	≥22	19-21.9	<19	≥22*	19-21.9*	<19*
Ciprofloxacin	CIP	5 µg/mL	≥20		<20	≥15		<15

Table contents include antibiotic information including concentration of antibiotic disks; these concentrations were as recommended in the EUCAST guidelines. Also included is published EUCAST sensitive/resistant breakpoints (available: http://www.eucast.org/clinical_breakpoints/) (EUCAST 2017).

*=no breakpoint published by EUCAST; breakpoint for other bacteria used, e.g. vancomycin breakpoint for *Enterococcus* spp. used for *S. aureus*.

3.2.10 McFarland standard

Originally described in 1907, the McFarland standard is a measurement of opacity indirectly representing bacterial numbers that is recommended for standardisation of microbiological testing (McFarland, 1907). However, more recently this method has been shown to result in large variation in microbial concentration between samples (Zapata and Ramirez-Arcos, 2015). Therefore, in an effort to standardise bacterial growth within this work, a 0.5 McFarland standard was prepared by mixing 0.6 mL of 1% (v/v) barium chloride ($BaCl_2$) with 99.4 mL of 1% (v/v) sulphuric acid (H_2SO_4). The attenuance of this was measured at 600 nm, with a value of 0.130 recorded against deionised water as a reference. Subsequently, for EUCAST testing, all isolates were grown to D_{600nm} = 0.130 before being applied to plates.

3.2.11 Antibiotics

A total of eight antibiotics were tested against the complete set of isolates in the biobank (Table 3.2). Antibiotics were chosen based on the recommendation of Dr David Farren (Consultant and Clinical Lead, Medical Microbiology, Northern Health and Social Care Trust) (Personal communication; Farren, 2016). We tested isolates against Cefoxitin (marker for Oxacillin and Methicillin resistance), Vancomycin, Penicillin, Erythromycin, Gentamicin, Ciprofloxacin and Fusidic Acid.

Table 3.2 – Overview of antibiotics used in antibiotic susceptibility testing of *Staphylococcus aureus* and *Enterococcus* spp. isolates recovered from hospital workers' uniforms.

Antibiotic	Class	Mode of action	Resistance mechanism
Cefoxitin*	β-Lactam (Cephamycin)	Inhibition of synthesis of bacterial walls – prevents crosslinking of peptidoglycan	Prevention of drug uptake, enzymatic modification and/or synthesis of beta-lactamases
Vancomycin	Glycopeptide	Inhibition of synthesis of bacterial walls – interferes with alanine-alanine bonds	Natural resistance – Gram negatives outer membranes prevents drug uptake. Some Gram positives don't need alanine-alanine bonds
Penicillin	β-Lactam (Penicillin)	Inhibition of synthesis of bacterial walls – prevents crosslinking of peptidoglycan	Prevention of drug uptake, enzymatic modification and/or synthesis of beta-lactamases
Erythromycin	Macrolide	Inhibition of protein synthesis – acts on 50S ribosomal subunit preventing protein elongation	Changes to 50S subunit to prevent drug binding and/or production of macrolide-digesting enzymes
Gentamicin	Aminoglycoside	Inhibition of protein synthesis – binds to 30S subunit causing mistranslation or loss of translation resulting in abnormal proteins	Prevention of drug uptake or production of drug degrading enzymes
Clindamycin	Lincosamide	Inhibition of protein synthesis – binds to 50S subunit prevention protein elongation	Structural changes to prevent drug binding
Fusidic acid	Fusidane	Inhibition of protein synthesis – inhibition of elongation factor G	Alteration of drug binding site and/or protection of drug binding site
Ciprofloxacin	Fluoroquinolone	Inhibition of nucleic acid synthesis – inhibits DNA gyrase needed for DNA replication	Binding site mutations reducing drug uptake

Information collated from Collignon and Turnidge, 1999, Chen *et al.,* 2010 and Bauman, 2013.

*Cefoxitin is used as a marker of methicillin and/or oxacillin resistance (U.S. Centers for Disease Control and Prevention, 2014). It is used as accurate determination of methicillin/oxacillin resistance rather than methicillin or oxacillin, as heteroresistance can occur in presence of methicillin or oxacillin. Cefoxitin is also a more effective inducer of the *mecA* gene (MRSA indicator), and thus leads to increased discrimination of results in disc diffusion assays (U.S. Centers for Disease Control and Prevention, 2014).

3.2.12 Multiple Antibiotic Resistance Indexes

The Multiple antibiotic resistant index (MAR index) is a numerical value representative of the proportion of tested antibiotics an isolate is resistant to (i.e. 1 = resistance to 100% of antibiotics tested, 0.5 = resistance to 50% of tested antibiotics; 0 = resistance to 0% of antibiotics tested). Using the antibiograms produced for uniform biobank isolates a MAR index was calculated for each isolate. MAR index values were calculated *(a/b, where 'a' represents the number of antibiotics the isolate was resistant to, and 'b' the total number of antibiotics the isolate was tested against)* for all isolates (Blasco et al., 2008).

3.3 Results

3.3.1 Protocol development/optimisation – Direct plating or Pre-enrichment

In order to determine the influence pre-enrichment has on enumeration of bacteria from uniforms in comparison with direct plating we tested one pre-shift and one post-shift garment using both direct plating and pre-enrichment steps before enumeration of microorganisms. Figure 3.2a shows the results of the direct plating testing and Figure 3.2b shows the results of the pre-enrichment testing. Table 3.3 summarises the results.

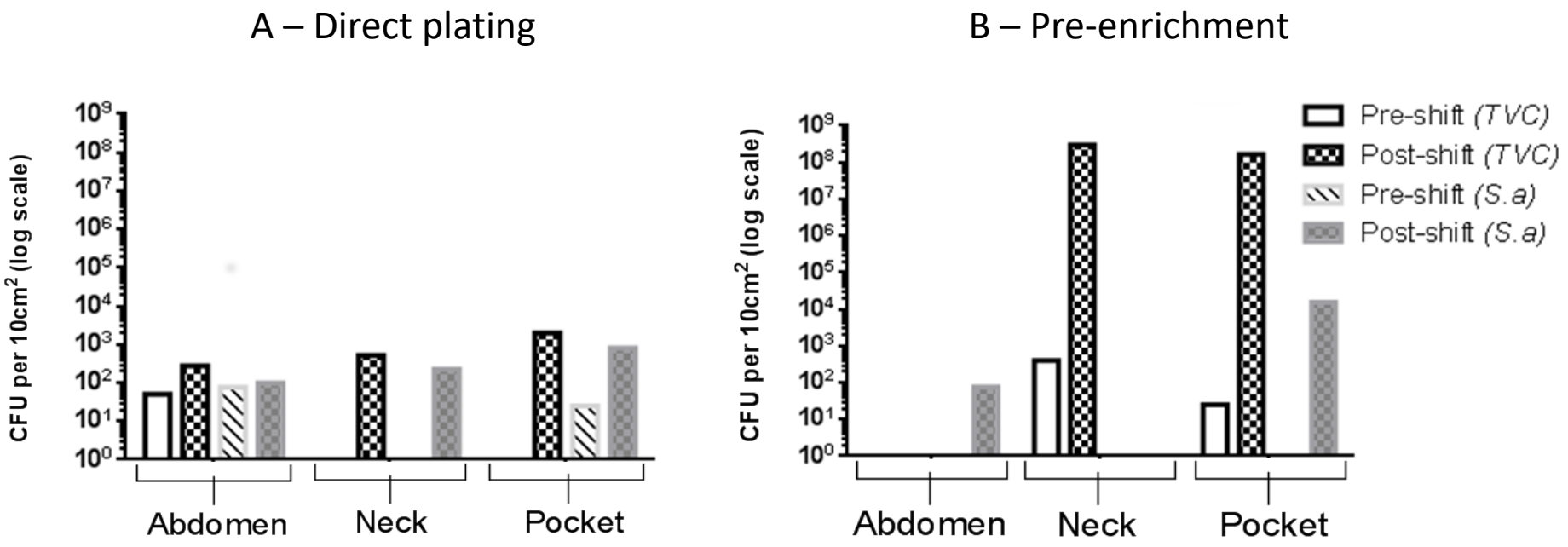

Figure 3.2 – Enumeration of total viable count and *Staphylococcus aureus* contamination of healthcare workers uniforms using the (A) direct plating method and (B) pre-enrichment method post sampling of uniforms. Data representative of mean per 10cm^2 area. Results are indicative of three areas (neck, abdomen and pocket) of one pre-shift and one post-shift uniform. Table 3.3 compares both methods.

Table 3.3 – -Comparison of Log^{10} changes between pre-shift and post-shift test uniforms when direct plating versus pre-enrichment.

TVC Log^{10} change			***Staphylococcus. aureus* Log^{10} change**		
Area	**Direct plating**	**Pre-enrichment**	**Area**	**Direct plating**	**Pre-enrichment**
Abdomen	0.74	0	**Abdomen**	0.12	1.88
Neck	2.72	5.87	**Neck**	2.35	0
Pocket	3.3	6.82	**Pocket**	1.5	4.89

This proof of concept experiment showed that bacterial contamination increased on the post-shift uniform on abdomen, neck and pocket areas, compared to levels determined on the pre-shift uniform. It is evident that the pre-moistened swab technique used is a sensitive recovery technique as bacteria were detected on all direct plating samples. Results for TVC and *S. aureus* contamination for pre-enrichment samples showed some exaggerated Log_{10} values. Log_{10} changes on pre-enrichment samples were up to 15-fold larger for *S. aureus* and 2 to 3-fold for TVC. Direct plating showed all samples were contaminated and demonstrated more modest Log^{10} changes. As the swabbing and direct plating recovery technique was sufficient to allow detection and accurate enumeration of bacteria (without potential basis of enrichment) these methods were used in subsequent work

3.3.2 Uniform collection

Uniforms were collected from Antrim Area Hospital, transferred to Ulster University, Coleraine for testing using the moistened swab, serial dilution and direct plating approach. Table 3.4 shows collection records for uniforms from Antrim Area Hospital. Uniform collection was documented and each uniform given a corresponding number according to the order in which they were sampled, for example post-shift uniform 1.

Table 3.4 – Collection of pre-shift and post-shift uniforms from Domestic Services at Antrim Area Hospital.

Date	**Pre-shift Uniforms collected (Uniform number)**	**Post-shift Uniforms collected (Collection group)**
3.5.16	1-5	1-5 (1)
4.5.16	6-10	6-10 (2)
10.5.16	11-20	11-20 (3)
13.5.16	21-30	21-30 (4)
17.5.16	31-40	31-40 (5)
23.5.16	41-50	41-50 (6)
3.6.16	51-70	*
17.6.16	71-90	*
20.7.16	*	51-70 (7)
22.7.16	91-100	71-80 (8)
28.7.16		81-100 (9)
Total number of Uniforms	**100**	**100**

* = Uniforms not collected on that day. All tested uniforms were documented and numbered according to the order they were sampled

3.3.3 Assessment of *Staphylococcus aureus* and *Enterococc*us spp. Bioburden of Healthcare Workers' Uniforms

A total of 100 pre-shift uniforms and 100 post-shift uniforms were assessed for contamination. *S. aureus* and *Enterococcus spp.* were recovered and subsequently enumerated from the abdomen, neck and pocket areas of each uniform resulting in a total of 600 samples. Pre-shift and post-shift numbers were compared to determine Log_{10} changes. Figures 3.3 and 3.4 show uniforms positive tests for *S. aureus* and *Enterococcus* spp. contamination on individual uniforms. Figure 3.5, Figure 3.6, Table 3.5 and Table 3.6 show quantitative information on levels of contamination. The data shows an increase in both *S. aureus* and *Enterococcus* spp. on post-shift uniforms compared to levels detected on pre-shift healthcare workers uniforms. There were only a very small number of positive pre-shift uniforms whereas most post-shift uniforms where contaminated. Pre-shift uniforms which were contaminated showed very low levels of contamination whereas there was a

1.89-2.84 Log_{10} increase on post-shift uniforms with the largest increases seen in the numbers of *S. aureus* recovered from neck area sample group.

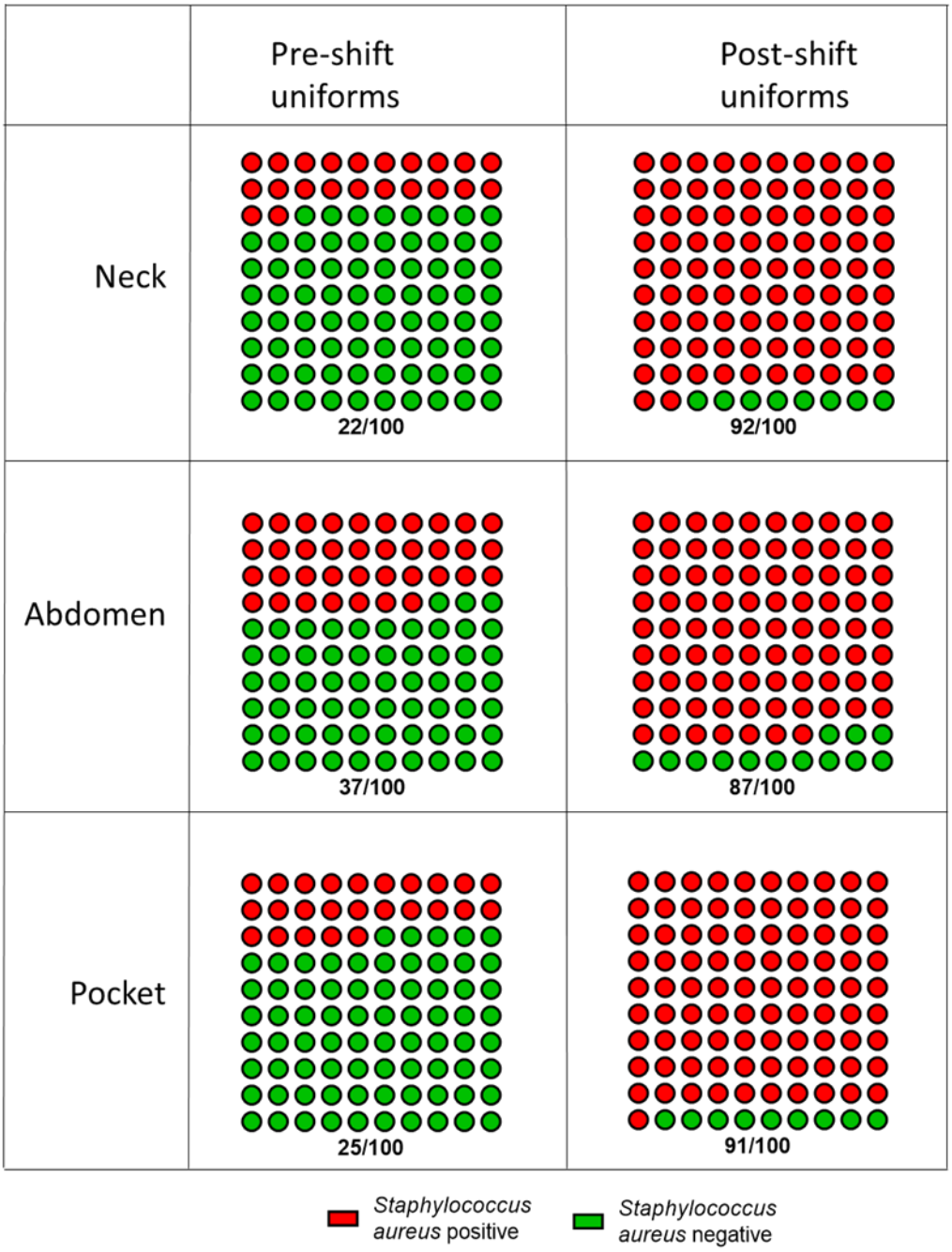

Figure 3.3 – Uniforms which tested positive for *Staphylococcus aureus* contamination. Numbers representative of 100 uniforms tested (n=100).

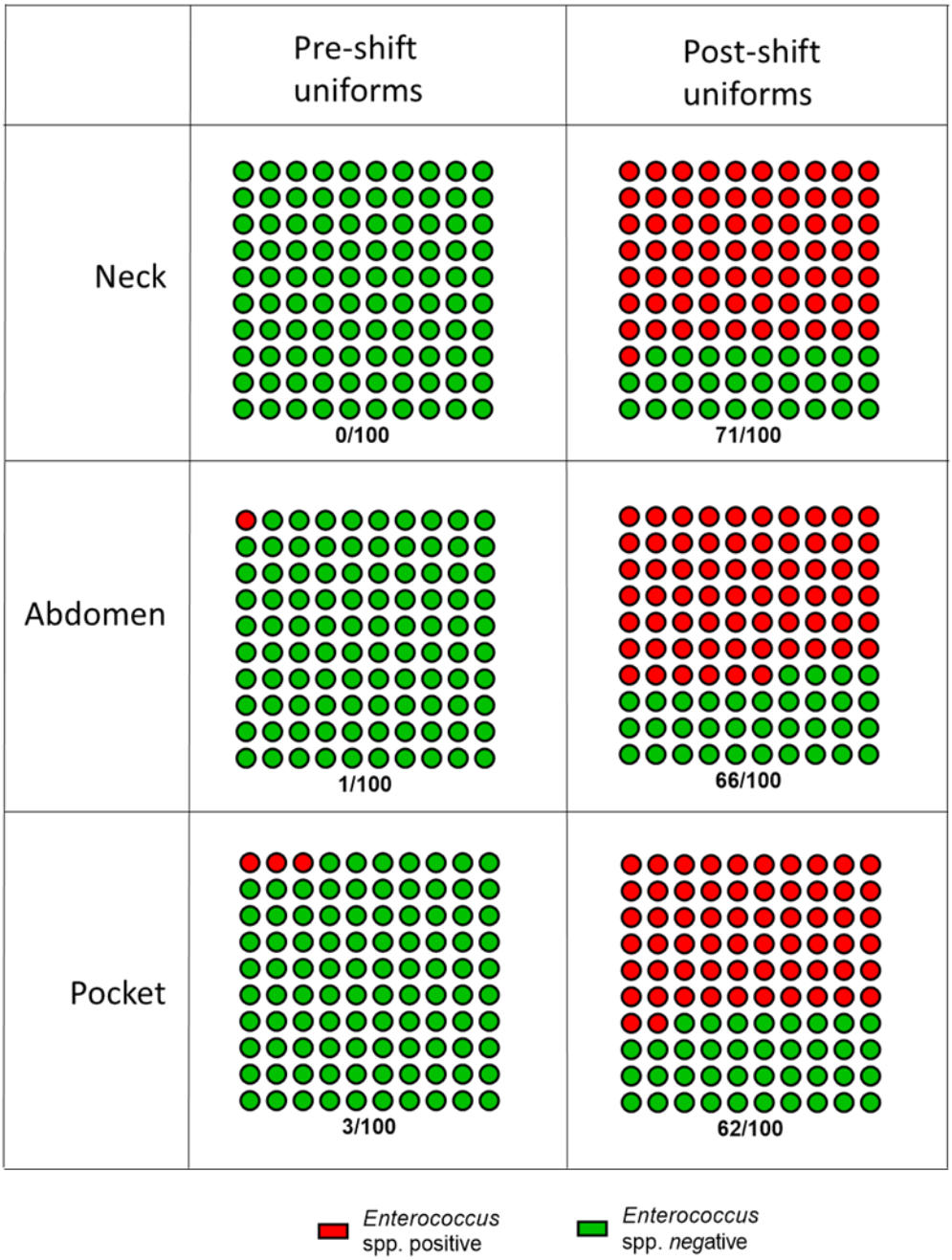

Figure 3.4 – Uniforms which tested positive for *Enterococcus* spp. contamination. Numbers representative of 100 uniforms tested (n=100).

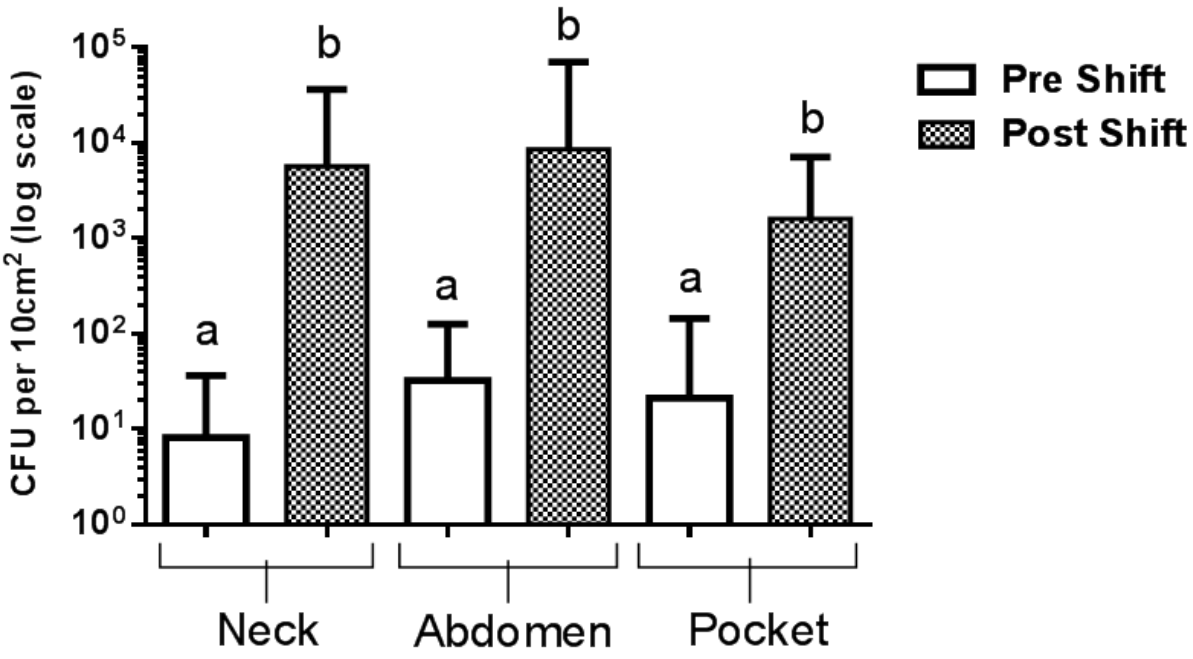

Figure 3.5 – *Staphylococcus aureus* contamination of pre-shift and post-shift healthcare workers uniforms. Data representative of mean CFU per 100 cm^2 +/- standard deviation (SD) (n=100). Statistical analysis using Wilcoxon test; data not sharing common subscript = $p<0.005$.

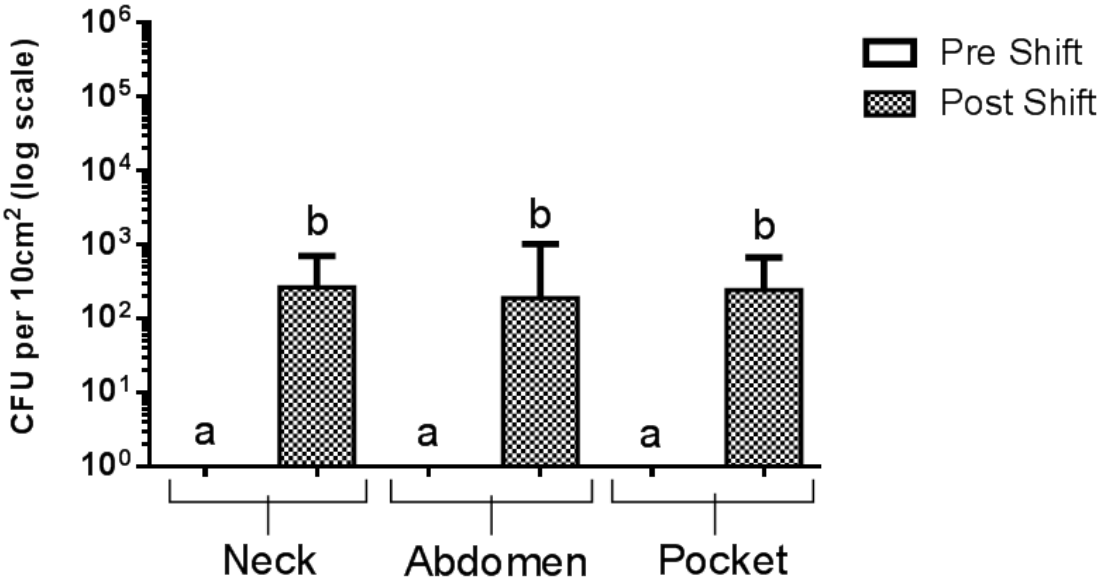

Figure 3.6 – *Enterococcus* spp. contamination of pre-shift and post-shift healthcare workers uniforms. Data representative of mean CFU per 100 cm^2 +/- standard deviation (SD) (n=100). Statistical analysis using Wilcoxon test; data not sharing common subscript = $p<0.005$.

Table 3.5 – Colony forming units per 100 cm^2 recovered from sampling sites pre and post shits, with Log^{10} changes for *Staphylococcus aureus*.

Staphylococcus aureus					
Area	**Pre shift**		**Post shift**		**Log10 change**
	Mean	**Standard Deviation**	**Mean**	**Standard Deviation**	
Neck	8.2	28.4	5721	30803	↑2.84
Abdomen	32.3	93.9	8616	62817	↑2.43
Neck	21.2	123.8	1606	5592	↑1.89

Value representative of CFU calculated from testing of 100 healthcare workers uniforms (n=100).

Table 3.6 – Colony forming units per 100 cm^2 recovered from sampling sites pre and post shits, with Log^{10} changes for *Enterococcus* spp. .

***Enterococcus* spp.**					
Area	**Pre shift**		**Post shift**		**Log10 change**
	Mean	**Standard Deviation**	**Mean**	**Standard Deviation**	
Neck	0	0	264.8	440.2	↑2.42
Abdomen	0.4	4	189.2	833.2	↑2.67
Neck	0.1	1.05	244.5	424.6	↑2.28

Value representative of CFU calculated from testing of 100 healthcare workers uniforms (n=100).

3.3.4 Antibiotic susceptibility testing of *Staphylococcus aureus* and *Enterococcus* spp. uniform isolates

S. aureus and *Enterococcus* spp. isolates randomly selected from tested post-shift uniforms were subject to antibiotic susceptibility testing using the EUCAST guidelines. Isolates were tested against 8 antibiotics commonly used in hospital laboratories, including Cefoxitin (a marker for MRSA) to determine MRSA prevalence amongst the *S. aureus* population and vancomycin to determine the VRE prevalence amongst the *Enterococcus* spp. population. Figures 3.7, 3.8, 3.9, 3.10, 3.11, 3.12 show antibiograms for these isolates. Antibiograms are categorised based on bacteria and the specific area of a given uniform that the bacterium was isolated from. Table 3.7 and Table 3.8 show prevalence of sensitive and resistant isolates against each antibiotic. MDR was only determined for *S. aureus* isolates and not for

Enterococcus spp. isolates as EUCAST provide official breakpoints for only 2 of the 8 tested antibiotics. Therefore, there was not a sufficient range of antibiotics to determine MDR for *Enterococcus* spp. isolates. However, in cases where no breakpoint was provided for either *S. aureus* or *Enterococcus* spp. the breakpoint for the corresponding bacteria was used to determine resistance or sensitivity for individual isolates. For example, no breakpoint is provided for vancomycin against *S. aureus*; therefore the *Enterococcus* spp. vancomycin breakpoint (12mm) was used to determine that antibiotic profile of *S. aureus* isolates.

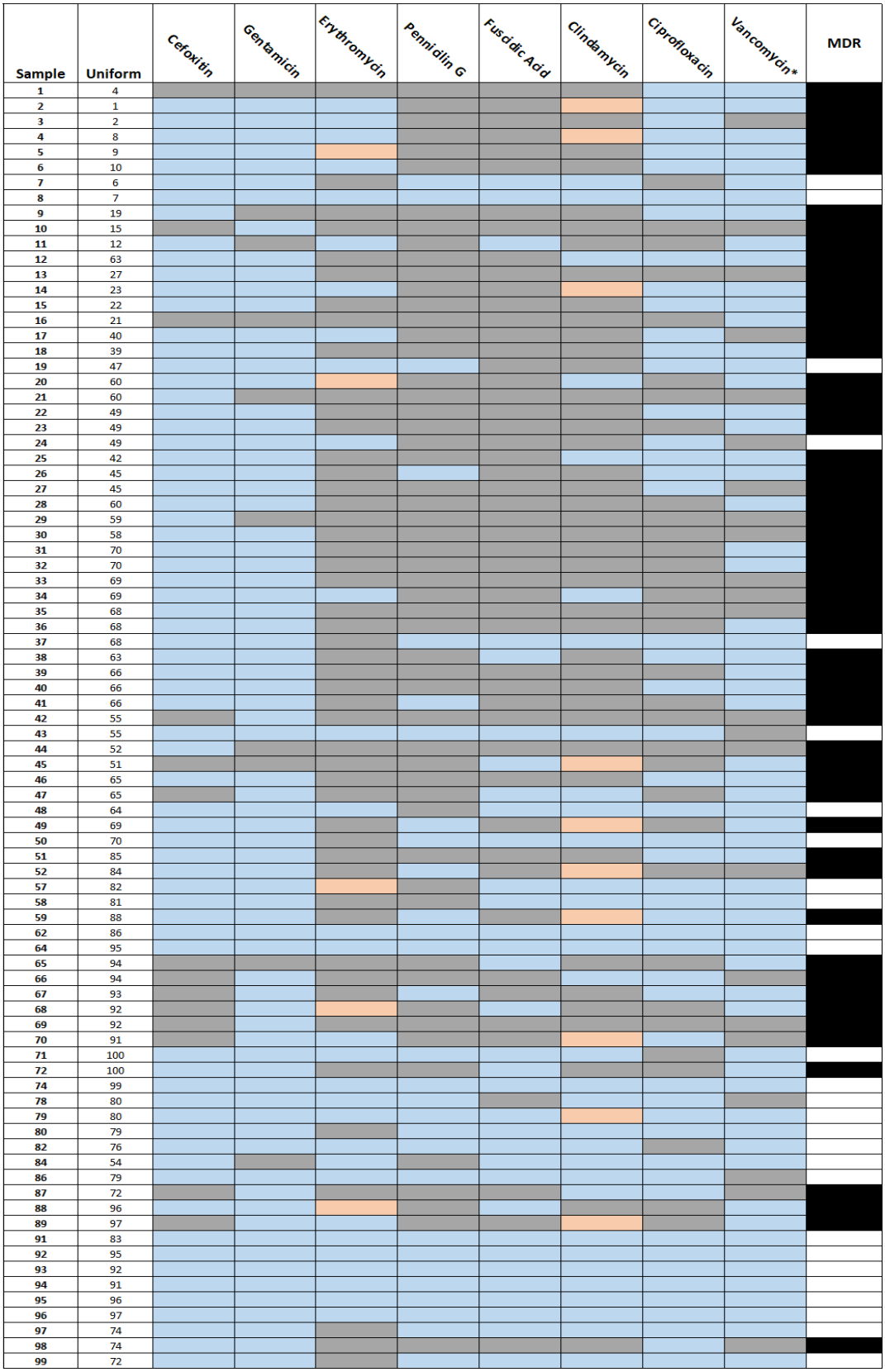

Figure 3.7 – Antibiogram for *Staphylococcus aureus* isolates from abdominal areas of post-shift uniforms. Blue = sensitive; grey = resistant; pink = intermediate resistance; black = Multi-drug resistant (MDR); white = non-MDR. *= *Enterococcus* spp. breakpoint used.

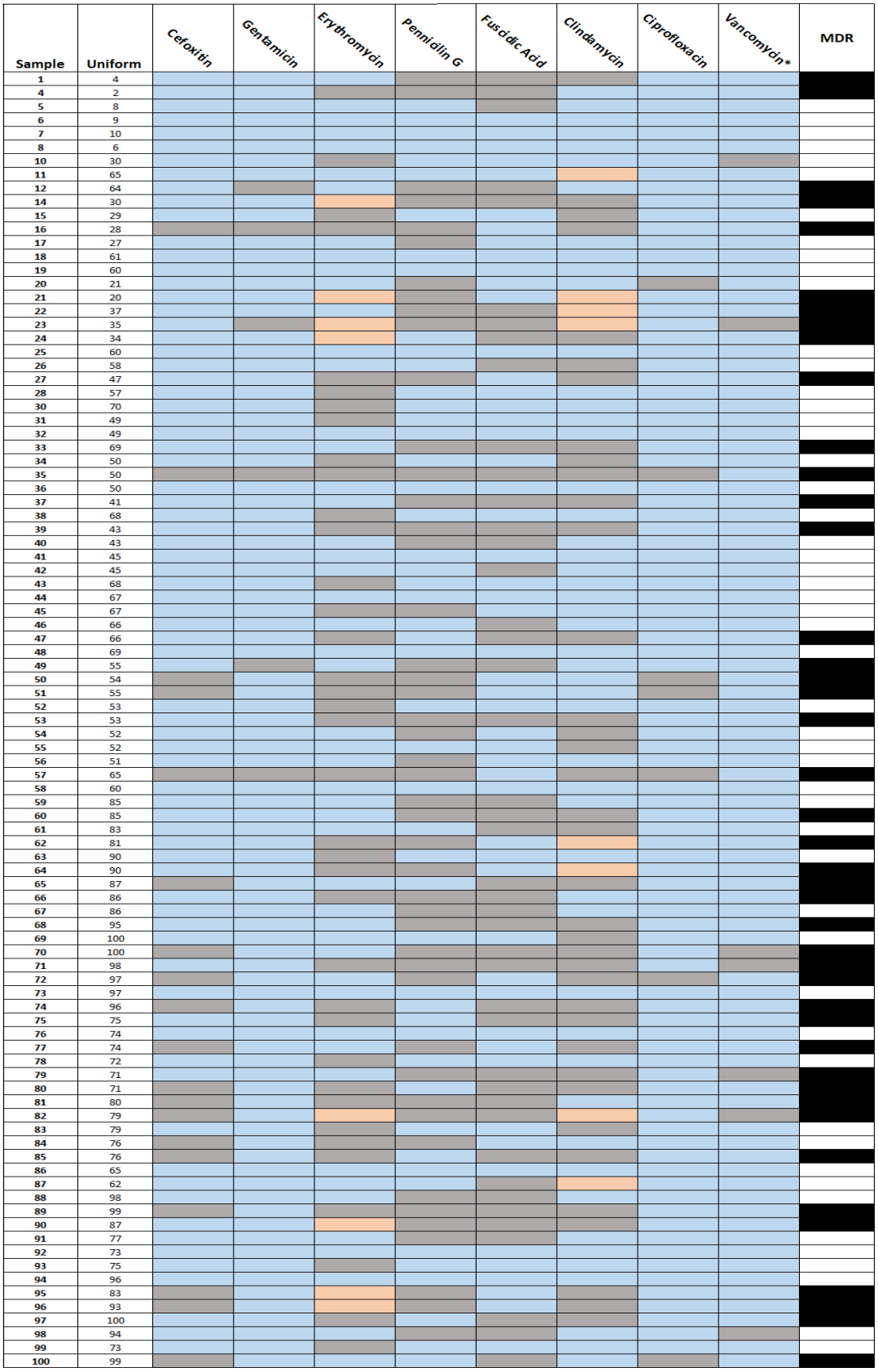

Figure 3.8 – Antibiogram for *Staphylococcus aureus* isolates from neck areas of post-shift uniforms. Blue = sensitive; grey = resistant; pink = intermediate resistance; black = Multi-drug resistant (MDR); white = non-MDR. *= *Enterococcus* spp. breakpoint used.

Figure 3.9 – Antibiogram for *Staphylococcus aureus* isolates from pocket areas of post-shift uniforms. Blue = sensitive; grey = resistant; pink = intermediate resistance; black = Multi-drug resistant (MDR); white = non-MDR. *= *Enterococcus* spp. breakpoint used.

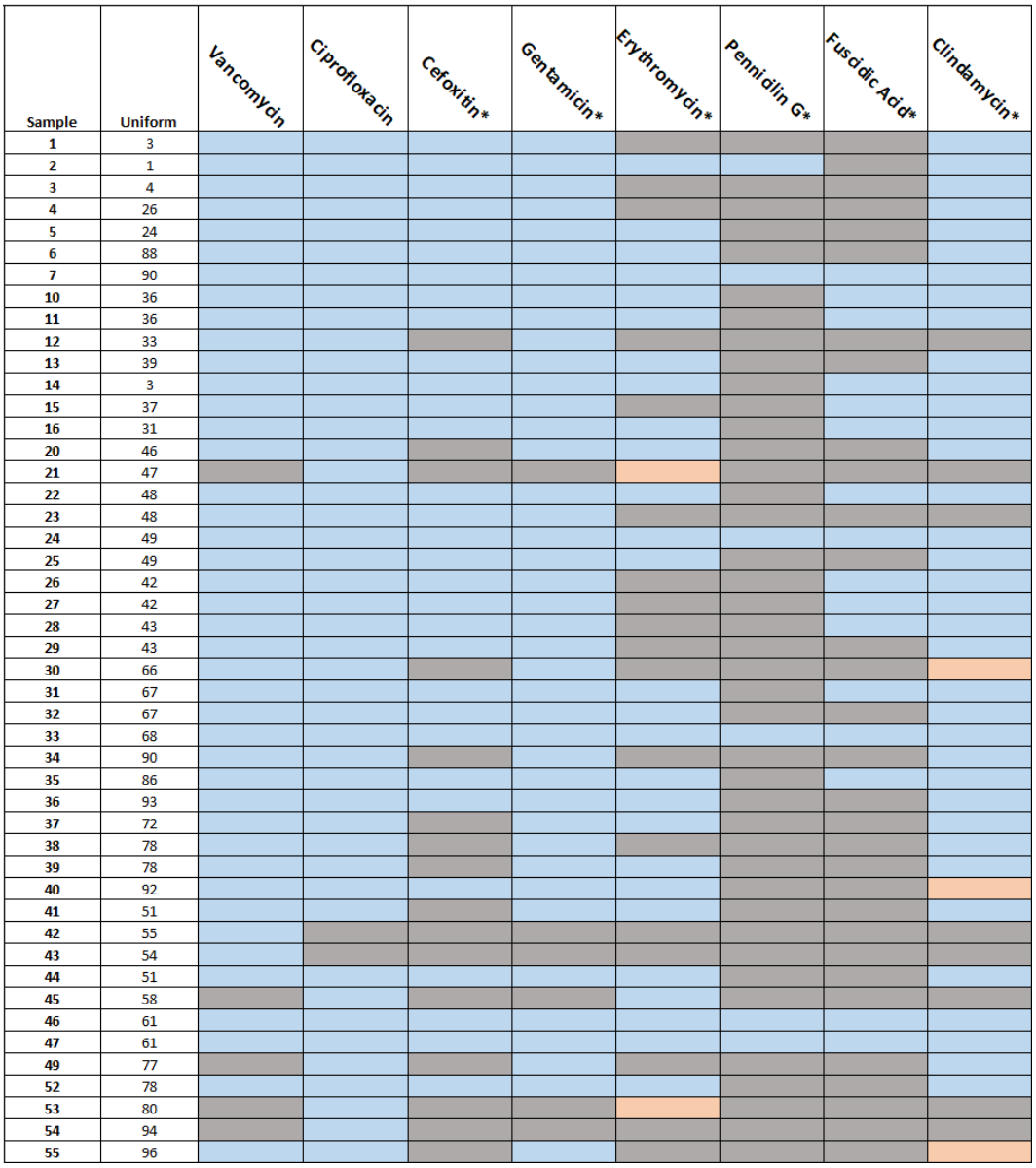

Figure 3.10 – Antibiogram for *Enterococcus* spp. isolates from abdominal areas of post-shift uniforms. Blue = sensitive; grey = resistant; pink = intermediate resistance; black = Multi-drug resistant (MDR); white = non-MDR. *= *Staphylococcus aureus* breakpoint used.

Sample	Uniform	Vancomycin	Ciprofloxacin	Cefoxitin*	Gentamicin*	Erythromycin*	Penicillin G*	Fusidic Acid*	Clindamycin*
1	3								
2	1								
3	4								
4	85								
5	83								
6	27								
7	74								
8	27								
9	74								
10	22								
11	82								
12	20								
13	18								
14	17								
15	99								
16	13								
17	9								
18	41								
19	36								
20	36								
21	36								
22	33								
23	33								
24	67								
25	67								
26	80								
27	40								
28	40								
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48	43								
49	71								
50	67								
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55	63								
56	63								
57	59								
58	53								
59	53								
63	57								
64	64								
69	71								
71	78								
72	79								
73	86								
76	90								
80	95								
81	95								
82	95								

Figure 3.11 – Antibiogram for *Enterococcus* spp. isolates from neck areas of post-shift uniforms. Blue = sensitive; grey = resistant; pink = intermediate resistance; black = Multi-drug resistant (MDR); white = non-MDR. *= *Staphylococcus aureus* breakpoint used.

Sample	Uniform	Vancomycin	Ciprofloxacin	Cefoxitin*	Gentamicin*	Erythromycin*	Penicillin G*	Fusidic Acid*	Clindamycin*
1	1								
2	29								
3	28								
4	27								
5	25								
6	24								
7	22								
8	21								
9	20								
10	61								
11	17								
12	9								
13	7								
14	33								
15	53								
16	68								
17	33								
18	34								
19	34								
20	36								
21	36								
23	31								
24	45								
25	45								
26	46								
27	46								
28	98								
29	90								
30	93								
31	93								
32	78								
33	80								
34	79								
35	54								
36									
37	54								
38	54								
39	59								
40	52								
41	52								
42	52								
43	61								
44	52								
45	53								
46	53								
47	55								
49	59								
53	66								
54	67								
59	79								
60	80								
61	82								
63	89								

Figure 3.12 – Antibiogram for *Enterococcus* spp. isolates from pocket areas of post-shift uniforms. Blue = sensitive; grey = resistant; pink = intermediate resistance; black = Multi-drug resistant (MDR); white = non-MDR. *= *Staphylococcus aureus* breakpoint used

Table 3.7 – Summary of resistance prevalence for *Staphylococcus aureus* isolates recovered from hospital workers' uniforms.

Antibiotic	*Staphylococcus aureus*											
	Abdominal (n=84)			Neck (n=95)			Pocket (n=85)			TOTAL (n=264)		
	S	I	R	S	I	R	S	I	R	S	I	R
Cefoxitin	83%	-	17%	80%	-	20%	78%	-	22%	80%	-	20%
Vancomycin*	73%	-	27%	93%	-	7%	90%	-	10%	85%	-	15%
Penicillin G	35%	-	65%	52%	-	48%	43%	-	57%	43%	-	57%
Erythromycin	36%	6%	58%	53%	8%	39%	55%	4%	41%	48%	6%	46%
Gentamicin	88%	-	12%	94%	-	6%	90%	-	10%	91%	-	9%
Fusidic acid	39%	-	61%	55%	-	45%	66%	-	34%	53%	-	47%
Clindamycin	38%	12%	50%	53%	8%	39%	64%	11%	25%	52%	10%	38%
Ciprofloxacin	61%	-	39%	93%	-	7%	65%	-	35%	73%	-	27%
Total MDR	56/84 (67%)			43/95 (45%)			35/85 (41%)			134/264 (51%)		

Numbers based on European Union Committee for Antibiotic Susceptibility Testing (EUCAST) analysis of isolates recovered from post-shift healthcare workers uniforms. Antibiotic profile (sensitive, resistant or intermediate resistant) determined from zone of inhibition averages (technical duplicates) compared to pre-defined breakpoints. *= No *S. aureus* breakpoint available, *Enterococcus* spp. breakpoint used to determine profile. S = sensitive; I = intermediate resistance; R = resistant.

Table 3.8 – Summary of resistance prevalence for *Enterococcus spp.* isolates recovered from hospital workers' uniforms.

Antibiotic	*Enterococcus* spp.											
	Abdominal (n=47)			Neck (n=69)			Pocket (n=53)			TOTAL (n=169)		
	S	I	R	S	I	R	S	I	R	S	I	R
Cefoxitin*	66%	-	34%	65%	-	35%	58%	-	42%	63%	-	37%
Vancomycin	89%	-	11%	83%	-	17%	87%	-	13%	86%	-	14%
Penicillin G*	13%	-	87%	20%	-	80%	13%	-	87%	15%	-	85%
Erythromycin*	57%	5%	38%	29%	0%	71%	30%	2%	70%	39%	2%	59%
Gentamicin*	87%	-	13%	84%	-	16%	83%	-	17%	85%	-	15%
Fusidic acid*	34%	-	66%	30%	-	70%	34%	-	66%	33%	-	67%
Clindamycin*	77%	6%	17%	64%	6%	30%	58%	4%	38%	66%	5%	29%
Ciprofloxacin	96%	-	4%	90%	-	10%	77%	-	23%	88%	-	12%

Numbers based on EUCAST analysis of isolates recovered from post-shift healthcare workers uniforms. Antibiotic profile (sensitive, resistant or intermediate resistant) determined from zone of inhibition averages (technical duplicates) compared to pre-defined breakpoints. *= No *Enterococcus* spp. breakpoint available, *S. aureus* breakpoint used to determine profile. S = sensitive; I = intermediate resistance; R = resistant.

3.3.5 Multiple Antibiotic Resistance Index Values for *Staphylococcus aureus* Isolates

MAR index values were calculated for each *S. aureus* isolate based on the antibiotic susceptibility profile determined by antibiotic sensitivity testing. This data was used to generate a reverse cumulative distribution plot as shown in Figure 3.13. None of the *S. aureus* isolates had an MAR index of 1 meaning that no isolates were resistant to all the antibiotics tested. Approximately 55-80% of isolates had a MAR index greater than 0 and approximately 20-55% of isolates had a MAR index 0.5 meaning they were resistant to half of the antibiotics tested.

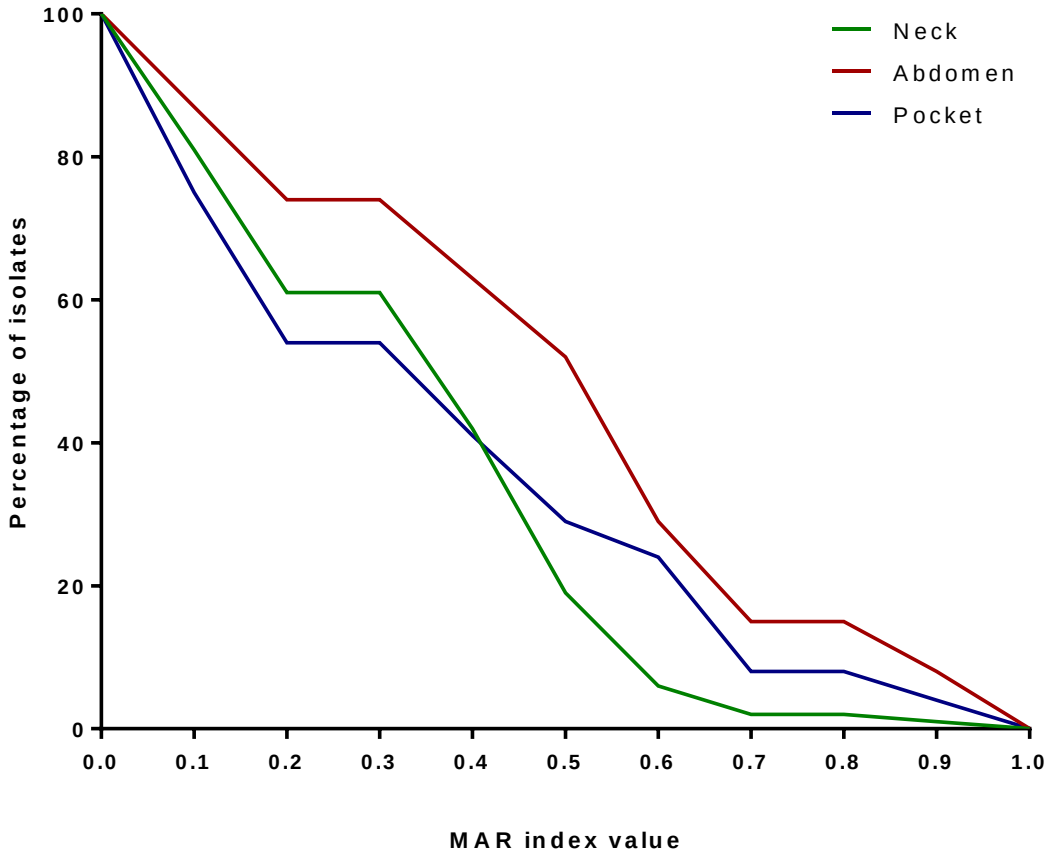

Figure 3.13 – Reverse cumulative distribution plot of MAR index values for *Staphylococcus aureus* uniform isolates. Data points representative of percentage of isolates equalling corresponding MAR index value or less. Graph generated using Graphpad Prism (neck n=95; abdomen n=84; pocket n=85).

3.3.6 Multiple Antibiotic Resistance Index Values for *Enterococcus spp.* Isolates

MAR index values were calculated for each *Enterococcus* spp. isolate based on antibiotic susceptibility profile determined by antibiotic sensitivity testing. This data was used to generate a reverse cumulative distribution plot as shown in Figure 3.14. Less than 5% (and only those isolated from the neck) of *Enterococcus* spp. neck isolates had a MAR index of 1 meaning they were resistant to all antibiotics tested. Approximately 90-95% of isolates had a MAR index greater than 0 and approximately 30-50% of isolates had a MAR index 0.5 across the three sampling sites meaning they were resistant to half of the antibiotics tested against.

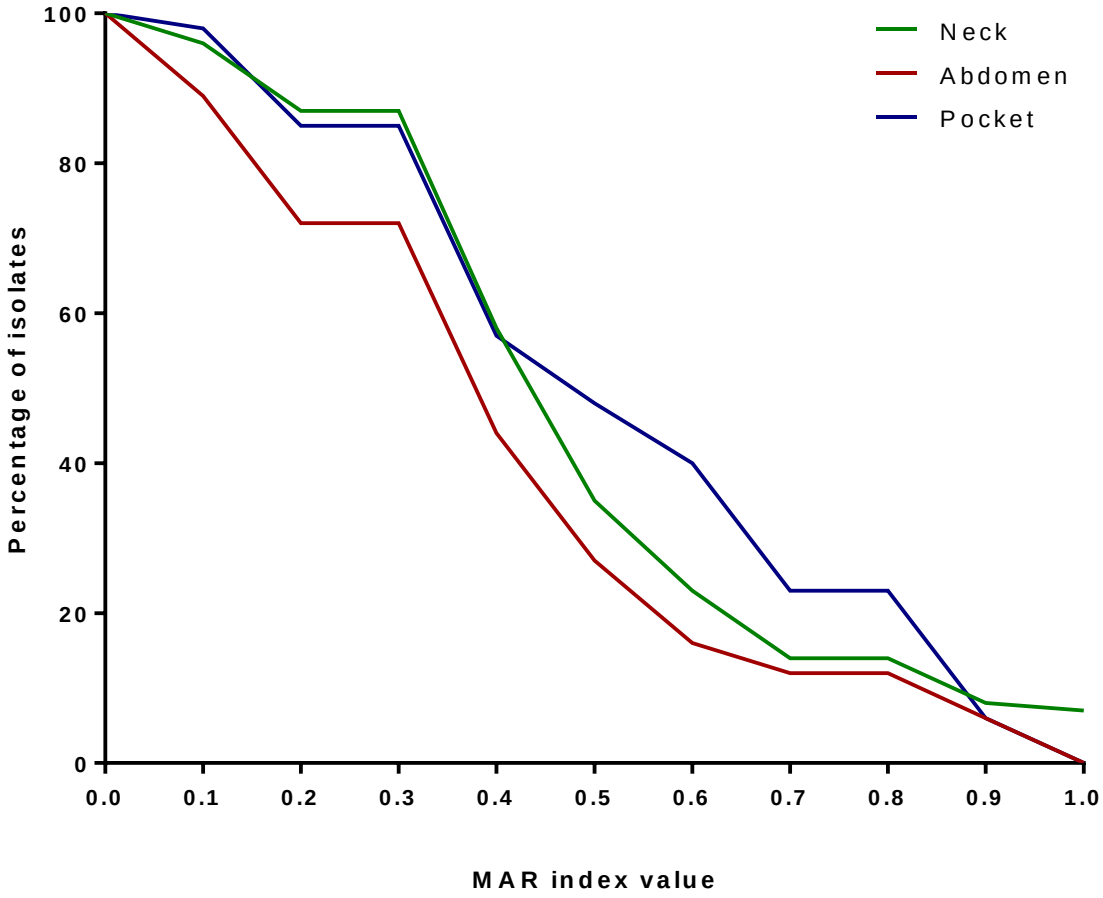

Figure 3.14 - Reverse cumulative distribution plot of MAR index values for *Enterococcus* spp. uniform isolates. Data points representative of percentage of isolates equalling corresponding MAR index value or less. Graph generated using Graphpad Prism (neck n=69; abdomen n=47; pocket n=53).

3.4 Conclusion

This work has shown that healthcare workers uniforms become contaminated with *S. aureus* and *Enterococcus* spp. during a normal working shift. This suggests strongly that environmental contamination is contributing to contamination of uniforms and that this contamination has the potential to act as an indirect route of transmission for highly pathogenic bacteria to vulnerable patients. Furthermore, it is evident that a proportion of these *S. aureus* and *Enterococcus* spp. isolated from post-shift healthcare workers uniforms are antibiotic resistant and a sub-population are classed as MDR.

3.4.1 Detection, Recovery and Enumeration of Bacteria from Uniforms

One of the aims of this work was "*Development of a detection, recovery and enumeration protocol for highly sensitive recovery of bacteria from fabric surfaces*". In the enumeration of bacteria from uniforms numbers as high as ~1×10^4 CFU were recovered for *S. aureus* and ~1×10^3 CFU for *Enterococcus* spp. These values allowed presentation in a Log_{10} scale which is important in bioburden studies as often Log_{10} changes are discussed to allow quantification of bioburden or the effects of an intervention.

CSA was used for isolation of *S. aureus* from uniforms. CSA is a chromogenic selective agar used for isolation of *S. aureus*, with *S. aureus* isolates producing pink/mauve colonies following incubation whereas other bacteria are inhibited or will appear blue, white or beige. Gaillot *et al*. (2000) tested the sensitivity of CSA for growth/identification of CSA in a comparison study with conventional methods of *S. aureus* isolation and found CSA to have significantly increased sensitivity (95.5% compared to 81.9%) (Gaillot et al., 2000). Other

groups have also evaluated the sensitivity of CSA with positive outcomes; Goodwin and Pobuda (2009) reported as high as 99% sensitivity (Goodwin and Pobuda, 2011), and similarly Han et al (2007) reported 98% sensitivity (Han et al., 2007), more examples can be found (Carricajo et al., 2001; Perry et al., 2003). For agar growth of presumptive *Enterococcus* spp*.,* Slanetz and Bartley agar (Oxoid, UK) was used. Slanetz and Bartley is described as highly selective for *Enterococcus* spp. at 44-45°C by the manufacturer/supplier. Originally described by Slanetz *et al.* (1955) as selective for *Enterococcus* spp. in water samples and verified by the same group in 1957 (Slanetz, *et al*.,1955; Slanetz and Bartley, 1957), it has also been used more recently for selective growth and enumeration of *Enterococcus* spp. from contaminated medical devices (Messina *et al.*, 2013) and from livestock environments (Agga *et al.*, 2015).

In comparison of the developed protocol with a study conducted in the same setting (Antrim Area Hospital) this work package demonstrates a better, more sensitive method for recovery of bacteria from uniforms. Johnston (2012) assessed uniforms for the effect of antimicrobial technology (AMI) on bioburden. The methods differed in sampling technique; here we used a swabbing method followed by recovery in diluent and subsequent serial dilutions and plating, whereas Johnston (2012) simply used a contact plate method where plates were contacted to the surface for 30 s. Other variations in the methods includes transport/sampling time (this chapter = 2 h; Johnston = 14 h), media used and number of uniforms tested. A direct comparison can be made between *S. aureus* recovery of post-shift uniforms (this chapter) and non-AMI uniforms (Johnston). The method described in this chapter demonstrated 250-fold increase in *S. aureus* numbers compared to that reported by Johnston (2012). In conclusion the method reported here allowed high sensitivity recovery and enumeration of bacteria from healthcare workers uniforms to accurately determine

Log_{10} differences between sample groups, i.e. pre-shift and post-shift. A comparison between both studies conducted at Antrim Area Hospital is summarised in Table 3.9.

Table 3.9 – Comparison of bioburden uniform studies conducted at Antrim Area Hospital.

	This Work		**Johnston, 2012**	
Location	Antrim Area Hospital		Antrim Area Hospital	
Sample groups	Pre-shift, Post-shift		Non-Antimicrobial, Antimicrobial	
Area tested	Neck, Abdomen and Pocket		Chest, Abdomen and Thigh	
Number of uniforms	200		257	
Number of samples	600		771	
Transport time	Less than 2h of direct collection		12h storage, 2h transport	
Sampling Method	Swab, serial dilutions, plating		Contact plates, 30s	
Area tested	100cm^2		25cm^2	
Media	CHROMAgar *S. aureus,* Slantz and Bartley		Nutrient agar, Baird-Parker agar, Enterococcosel	
Maximum Recovery				
	This Work		**Johnston, 2012**	
	S. aureus	*Enterococcus* spp.	*S. aureus*	*Enterococcus* spp.
Pre-shift (Fresh uniforms)	~10 cfu	0	0	0
Post-shift (Standard suits)	~10,000 cfu	~1000 cfu	~40 cfu*****	0

*number adjusted (multiplied by 4) to normalise area tested to our work.

3.4.2 Contamination of Healthcare Workers' Uniforms

Pre-shift and post-shift healthcare workers uniforms' were assessed for *S. aureus* and *Enterococcus* spp. contamination. Levels of both *S. aureus* and *Enterococcus* spp. contamination were significantly increased in post-shift uniforms compared to pre-shift uniforms. This indicates that contamination of the uniforms occurs during a working day within the vicinity of the hospital. This increase in contamination levels on uniforms is confirmation of environmental contamination within hospital, which poses risk of direct transmission to patients and which could potentially cause infection. Moreover, the microbial contamination found on uniforms could also be transmitted to patients (indirect transmission). Our data showed considerable increases in levels of contamination on healthcare workers' uniforms and indeed other studies have shown similar types of bacteria on healthcare workers hands and uniforms (Kramer *et al.* 2006); this work provides further evidence that healthcare workers become contaminated with potentially pathogenic bacteria.

In comparison with similar work in the literature this work further confirms contamination of healthcare workers uniforms. Some of the previously discussed studies had also shown bacterial contamination of uniforms. (Gaspard *et al.*, 2009; Johnston, 2012; Abu Radwan and Ahmad, 2017). More specific examples include a study by Sanon (2012) where pre-sterilised uniforms were provided to a small sample group of staff and all uniforms became contaminated during a work shift by an average of 5,795 CFU per square inch – some of the bacteria isolated included MRSA*, S. epidermidis, Bacillus* sp. amongst others (Sanon, 2012). This was a much smaller sample group (11 uniforms) however traceability was included as the same pre-sterilised uniform was tested after use (Sanon, 2012). Another example where pre-shift and post-shift uniforms were tested had shown minimal levels of contamination of

uniforms pre-shift however the authors had detected MRSA, VRE and *C. difficile* on an increased number of uniforms post shift (Perry *et al.,* 2001). Despite this, however only detection of bacteria was reported in contrast with the work in this chapter where we both detected and enumerated bacterial levels.

3.4.3 Antibiotic Resistance in the Hospital Environment

The biobank of *S. aureus* and *Enterococcus* spp. isolates collected in this work was tested for antibiotic susceptibility against 8 antibiotics which are used in testing with Antrim Area Hospital (Farren, 2016, *pers comm.*). As hypothesised, antibiotic resistant and MDR bacteria were present. Some 14% of *Enterococcus* spp. isolates were vancomycin resistant (thus VRE), a figure which correlates well with a study by Rengaraj *et al* (2016) in a teaching hospital where 12.9% of *E. faecalis* strains were identified as VRE by the disc diffusion method. Ireland is unique in having the highest prevalence of VRE cases in Europe. Ryan *et al.,* (2015) reported that 45% of *E. faecium* isolated from blood cultures was also VRE. Thus, whilst numbers in this study are significantly lower (14%), we assessed total *Enterococcus* spp. rather than *E. faecium* specifically.

An emerging issue is vancomycin resistant *S. aureus* (VRSA) as vancomycin is often used for treatment of MRSA infections however resistance has been noted. Within the Ulster biobank collection of *S. aureus* isolates, 20% were classed as MRSA and 15% as VRSA. However, only 5% were both MRSA and VRSA. Hiramatsu *et al*. (1997) previously reported that 1.3-20% of >1000 MRSA isolates to be VRSA within several hospitals (Hiramatsu *et al.*, 1997), however more recently one study isolating *S. aureus* from burn patients reported that ~39% of MRSA were also VRSA (Hasan *et al.,* 2016). The numbers we report are lower than these seen in

the literature with regard to MRSA/VRSA, however some of these studies assess patient/blood cultures which are likely to be more resistant than those isolated from healthcare workers' uniforms.

Within the collection, significantly 51% of *S. aureus* isolates were classed MDR and 100% of MRSA isolates were MDR. This is defined as being resistant to one agent in at least three anti-microbial groups. The average MAR index values were 0.29 for *S. aureus* and 0.4 for *Enterococcus* spp. This global representation of antibiotic resistance data indicates a high level of antibiotic resistance. The presence of these highly pathogenic antibiotic resistant bacteria is of considerable concern as there is well documented evidence of high risk of infection of patients, which in turn will increase morbidity and mortality rates, leading to extra strain on resources including increased costs (Hardy *et al.*, 2006; Goodwin and Pobuda, 2011; Cheng *et al.*, 2015; O'Neill, 2016).

Additionally, the presence of these antibiotic resistant bacteria means the presence of antibiotic resistant genes in the hospital environment. Therefore, there is the potential for increased emergence of antibiotic resistance, whether that derives from intrinsic development of antibiotic resistance (via antibiotic-driven selection) or by gene uptake mechanisms such as horizontal gene transfer of these mobile genetic elements from a resistant bacterium onto a sensitive bacterium (Davies, 1994). These recurring processes are shown in Figure 3.15 (Davies, 1994). Rowe *et al.* (2017) have shown that there are significantly increased levels of antibiotic resistance genes (including β-lactam resistance genes) from a hospital effluent in comparison with the outflow from the surrounding residential areas (Rowe *et al.*, 2017). Similarly, Rodríguez-Mozaz *et al.* (2015) assessed wastewater from a hospital and showed the presence of both antibiotics *and* antibiotic

resistance genes for fluoroquinolones, β-lactams, macrolides, sulphonamides and tetracyclines (Rodriguez-Mozaz *et al.*, 2015). The presence of these antibiotic resistant bacteria on uniforms tested at Antrim Area Hospital could therefore potentially contribute to an antibiotic resistance gene pool and could potentially result in increased antibiotic resistance.

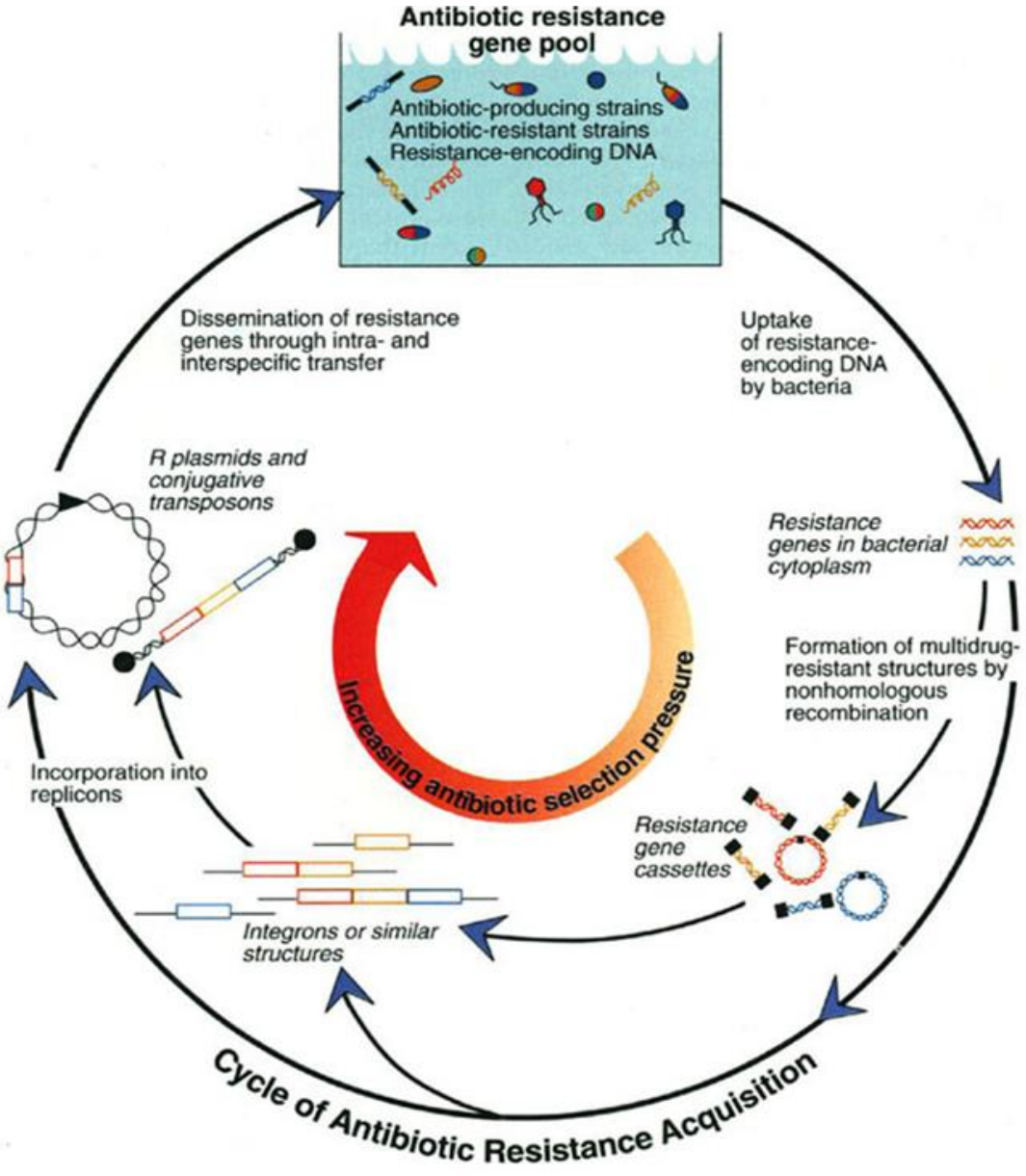

Figure 3.15 – Presence of antibiotic resistance genes in an environment could potentially result in sensitive bacteria acquiring antibiotic resistance by gene transfer mechanisms (taken from Davies, 1994).

3.4.4 Summary of Findings

- In this work we developed a high sensitivity protocol that allows rapid and accurate detection and enumeration of bacteria from fabric surfaces.
- Large increases (2-3 Log^{10}) in *S. aureus* and *Enterococcus* spp. contamination on post-shift healthcare workers uniforms indicating environmental contamination leading to contamination of uniforms – with the possibility for direct and indirect transmission routes of HAIs onto patients.
- The isolates collected during the work exhibited antibiotic resistance, and multi-drug resistant bacteria were detected on uniforms. Thus, we conclude that there is:
    - Potential for transmission of highly pathogenic bacteria onto patients.
    - Increased presence of antibiotic resistance-encoding mobile genetic elements – could result in increased antibiotic resistance.

Chapter 4

Diversity Analysis of *Staphylococcus aureus* and *Enterococcus* spp. isolated from Healthcare Workers' Uniforms

4.1 Introduction

4.1.1 Surveillance of Healthcare Acquired Infections

Infection control plays a vital role in the prevention of HAIs by eradicating/minimising microbial contamination in the healthcare environment. An important aspect of infection control practices is monitoring or surveillance of the microorganisms in hospitals (Sydnor and Perl, 2011). One of the first infection control programs was the Study on The Efficacy of Nosocomial Infection Control (SENIC Project) authorised by the CDC (Haley *et al.*, 1980; Quade *et al.*, 1980; Hughes, 1988).

Surveillance is *"the ongoing and systematic collection, collation, and analysis of data, and the dissemination of the results to those who need to know to avoid or prevent infections or epidemics"* (Nelson and Williams, 2014). Surveillance is common element of infection control guidelines published by hospitals (Health Protection Scotland, 2014; Public Health England, 2017). Researchers also commonly conduct large scale surveillance of HAIs, for example Li *et al.* (2018) monitored intensive care units in 176 hospitals in China for nosocomial infectious organisms consistent with presence of bacteria in the ICUs (Li *et al.*, 2018). Interestingly, Li *et* al. (2018) concluded in multiple publications that surveillance not only acts as an information tool but that during surveillance periods there are reduced HAI rates, most likely due to better adherence to infection control practices by healthcare workers (Li *et al.*, 2017, 2018). Other recent examples of research in the surveillance of microorganisms include a 10-year surveillance programme for VRE in German healthcare settings (Remschmidt *et al.*, 2018) and assessment of the occurrence of antibiotic resistant HAIs in Korean intensive care units (Choi *et al.*, 2016). These hospital guidelines and research

articles often conclude the importance of surveillance of microorganisms *"in order to develop proper strategies for preventing and treating nosocomial infections"* (Choi *et al.*, 2016).

Whist the traditional method of monitoring bacteria via direct analysis of the presence/absence of infectious agents (for example bioburden studies ((chapter 3) remains important – there is a growing role for epidemiological analysis (and typing) of HAI bacteria. These methods often identify, characterise or assess trends (for example antibiotic profiles) in HAIs (Nelson and Williams, 2014; Choi *et al.*, 2016).

4.1.2 Molecular Epidemiology for Surveillance of Healthcare Acquired Infections

Riley defined molecular epidemiology as *"the study of the distribution and determinants of infectious diseases that utilizes molecular biology methods"* (Riley, 2004). Molecular methods are often applied for epidemiological investigations and the surveillance of HAIs. This involves an experimental approach to rapidly assess the genotypic characteristics of bacteria to either identify or characterise a population of isolates, i.e. genotyping. This allows comparisons of HAIs with a high level of discriminatory power with multiple applications (Foxman, 2012). Molecular epidemiology tools can be used to distinguish microorganisms at a genus, species or strain level and hence may be used to determine causative agents of infections or outbreaks in healthcare settings, and to determine clonality or relatedness between populations of microorganisms of interest (Ramirez *et al.*, 2015). Furthermore, certain molecular methods could also provide information on pathogenicity, antimicrobial resistance, prevention and treatment options (Foxman, 2012; Ramirez *et al.*, 2015).

There are many methods available for use in molecular epidemiology of microorganisms and some of these will be discussed in subsequent sections. The ideal molecular tool would be whole genome sequencing (WGS) as this would allow the user the highest discriminatory power (theoretically could distinguish between a single nucleotide difference) and provide a wealth of information about the organism (Salipante *et al.*, 2015). However, alternative molecular tools utilise genetic variation to discriminate amongst members of a microbial population, i.e. to identify and determine genomic variants of a population and to conduct comparative analysis to determine relatedness. Examples of outputs of these techniques include tracking of bacteria either within a hospital (Leong *et al.,* 2018) or internationally (He *et al.,* 2013). For example Leong *et al.* (2018) used whole genome sequencing to map VRE movement throughout an Australian hospital, they were able to track the movement of specific sequence types of VRE throughout the hospital and characterise the genetic evolution of the bacteria as it moved through hospital departments by identifying single nucleotide polymorphisms. On an larger scale, He *et al.* (2012) used whole genome sequencing to track epidemic *C. difficile* 027 from Canada to the UK, mainland Europe and Australia, furthermore they tracked the movement nationally within the UK, eventually tracking its way to Stoke Mandeville where a serious outbreak of *C. difficile* resulted in numerous deaths.

4.1.3 Pulse-field Gel Electrophoresis (PFGE)

Pulse field gel electrophoresis (PFGE) (first described by Schwartz *et al.*, 1983) is a method of typing organisms based on “a genetic fingerprint” or banding pattern. The fingerprint is generated using specific restriction enzymes which digest DNA at specific target sites cleaving into a number of fragments (Simner *et al.,* 2015). This restriction digest is purposely designed to result in a small number of large size fragments (Wang *et al.,* 2015) which are

then subject to gel electrophoresis. Voltage is applied using alternating directions of the electric field to allow the user to resolve large DNA fragments. Various gel electrophoresis methods have been applied for this and the most common is referred to as contour-clamped homogenous electric field (CHEF) (Wang *et al.,* 2015; Parizad *et al.,* 2016). The banding pattern produced from the gel electrophoresis is considered a genomic fingerprint for the organism under investigation and can be used in comparative/relatedness epidemiological investigations. The process of PFGE involves suspending cells in melted agar and lyses of the cells for DNA release, to which a restriction digest enzyme is added. The sample is then subject to pulse field gel electrophoresis and visualised under UV light with a suitable stain to obtain a banding pattern (Briczinski and Roberts, 2007; Parizad *et al.,* 2016).

PFGE is often considered the 'gold standard' molecular epidemiology tool (Goering, 2010; Tibayrenc, Abdelbary, *et al.*, 2017). Engelhart *et al.* (2002) used PFGE to identify environmental contamination of surface cleaning equipment in a haematology-oncology unit. They showed that the PFGE pattern of *P. aeruginosa* isolated from infected patients matched the PFGE pattern of *P. aeruginosa* isolated from the cleaning equipment (Engelhart *et al.*, 2002). More recently, Kreidl *et al.* used PFGE to assess an outbreak of VRE and found identical PFGE patterns from patient isolates and from environmental contamination (Kreidl *et al.*, 2018). However, issues surrounding reproducibility and standardisation have been noted for PFGE, especially in regard to inter-laboratory investigations. Murchan *et al.* reported the importance of strict standardisation of many of the parameters in the PFGE protocol and during their assessment of a 'harmonised' protocol showed that patterns from some samples were not reproducible when assessed in different laboratories (Murchan *et al.*, 2003). Goering also reported intra-reproducibility and inter-reproducibility issues,

highlighting in particular standardisation problems with DNA extraction, restriction enzyme digestion and electrophoresis conditions (Goering, 2010).

4.1.4 Multi-Locus Sequence Typing (MLST)

Multi-locus sequence typing (MLST) is another tool that can be implemented in molecular epidemiology (Pérez-Losada *et al.*, 2011; Tibayrenc *et al.*, 2017). MLST was first used by Maiden *et al.* (1998) in assessment of *Neisseria meningitides.* They analysed 11 housekeeping gene sequences in a collection of 107 isolates, effectively creating the first MLST database (Maiden *et al.*, 1998). MLST analyses nucleotide sequences of 5-7 highly conserved housekeeping genes for an organism (Dingle and MacCannell, 2015). The genes of interest are standardised for organisms and found on http://www.mlst.net or http://www.pubmlst.org. As multiple sequences are analysed, the combination of alleles results in a sequence type (ST) for the organism of interest, STs are determined by analysis of sequences using the MLST database derived a ST for an organism (Chui and Li, 2015; Paris *et al.,* 2015). Within a population of bacteria, STs can be compared to determine relatedness of organisms based on allelic variation. The process of MLST is carried out by conducting PCR for MLST genes (pre-defined per organism) followed by sequencing of PCR products and using this data to determine a ST (Chui and Li, 2015). An alternative version of MLST, multi-virulence-locus sequence typing (MVLST) can also be used for epidemiological investigations and is based on the same PCR-sequencing principles but specifically analyses the sequences of virulence genes (Chui and Li, 2015).

Maiden *et al.* concluded that the use of MLST in epidemiological investigations was potentially valuable due to the high levels of standardisation as sequence data is generated

and used for analysis/comparisons (Maiden *et al.*, 1998). MLST has been used in epidemiological investigations, for example Yin *et al.* (2018) used MLST to assess 196 *P. aeruginosa* isolates from bloodstream infections and wounds of patients in a Chinese burn centre. They identified 58 STs and could correlate isolates with a previous ST (ST111) known to have been the causative agent of an outbreak in 2014 (Yin *et al.*, 2018). However, the application of MLST for molecular epidemiology is still debated. While the analysis of sequences generates standardised data, it subsequently reduces the discriminatory power especially in use for epidemic (local) outbreaks (Tibayrenc, *et al.*, 2017), due to the high levels of sequence conservation in the genes used for analysis. Isolates from a similar geographic population are thus unlikely to display great diversity in these housekeeping genes. On the other hand, MLST has high discriminatory power with regard pandemic (global) outbreaks for the same reasons (Wang *et al.,* 2015). Other limitations of MLST include cost and data analysis time constraints (Tibayrenc *et al.*, 2017).

4.1.5 Random Amplification of Polymorphic DNA (RAPD-PCR [RAPD hereafter])

Random amplification of polymorphic DNA (RAPD) is a molecular technique that uses PCR for DNA amplification of arbitrary sequences. RAPD was first described by Williams *et al.* (1990) as an alternative method for generating molecular genetic maps. Single primers of 5-10 bases are utilised which serve as both the forward and reverse primer in the PCR (Grody *et al.*, 2010) and less stringent conditions are used than those commonly used for PCR based techniques. Due to the low-stringency PCR conditions and non-specific short primers, many primer binding sites are available on template DNA resulting in strain-specific amplification of multiple fragments of varying size (Tang *et al.*, 2015). As only one primer is used amplification requires the primer to bind to opposite strands of the DNA, these binding

points must be in relatively close proximity to each other (100-3000bp) (Figure 4.1) for successful amplification (Hiett, 2011) and the distance between binding points also determines the PCR product length (Hata, 2010), thereby increasing the randomness of RAPD. Thus, variation in location and number of binding sites, coupled with low stringency PCR conditions results in variation of banding patterns and in bacterial populations to be detected (Hata, 2010). This is achieved by visualising amplified fragments using staining protocols and gel electrophoresis to generate a banding pattern/RAPD fingerprint for isolates. The process of RAPD includes DNA extraction, PCR, gel electrophoresis and comparative analysis of banding patterns. These banding patterns act as a 'fingerprint' for samples and can be used to determine genomic diversity in comparative analyses relatively easy. Figure 4.2 highlights how RAPD can provide different fingerprints for two independent DNA templates using the same primer (adapted from Arif et al. 2010).

RAPD has been used for molecular epidemiological investigation of infectious outbreaks in hospitals. Qi *et al.* (2018) assessed RAPD patterns of *Candida parapsilosis* isolated from infected neonatal patients and 313 samples isolated from the environment. Using RAPD they were able to determine the same RAPD pattern for environmental isolates and patient isolates (Qi *et al.*, 2018). This information was used to support an increase in infection control measures. Aditi *et al.* (2017) used RAPD to type 87 *P. aeruginosa* isolates, generating 71 RAPD fingerprints. However the analysis showed marked similarity (85%) amongst all isolates (Aditi *et al.*, 2017). In contrast to PFGE and MLST, RAPD requires no information on the genomic make up of an organism of interest (Carrascosa *et al.*, 2011) (MLST and PFGE requires sequence information for primer and restriction enzyme design), and is a cost effective, high throughput (Chifiriuc *et al.*, 2017) method for epidemiological/diversity analysis of a large population of bacteria. Despite this, reproducibility issues have been

highlighted with RAPD, due primarily to the randomness of the method, and also to intra-reproducibility, inter-reproducibility and inter-laboratory-reproducibility issues that are well documented (Carrascosa *et al.*, 2011; Hiett, 2011). A potential reason for this is that the low stringency PCR conditions result in unstable PCR fragments that can be difficult to reproduce (Carrascosa *et al.*, 2011).

In order to analyse the biobank of uniform bacteria isolated in chapter 3 we performed RAPD. The RAPD fingerprints allowed a diversity dendrogram to be produced to assess population diversity of the *S. aureus* and *Enterococcus* spp. isolates recovered from healthcare workers' uniforms. Control isolates were also assessed to determine RAPD reproducibility and develop a robust RAPD protocol to overcome reproducibility issues.

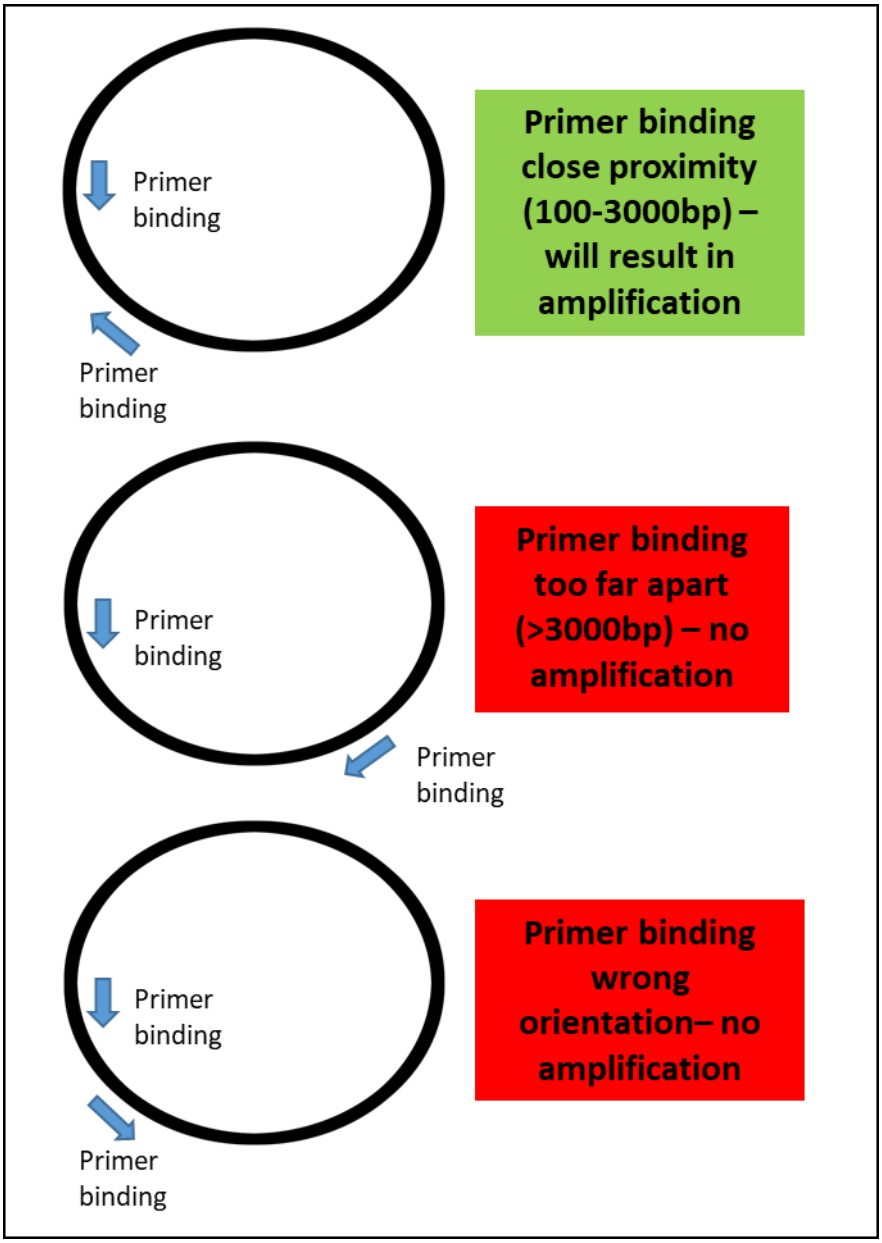

Figure 4.1 – Primer binding in RAPD. Primers must bind in the correct orientation in close proximity for successful amplification. If primers are too far apart and/or in the wrong orientation amplification will not occur during PCR.

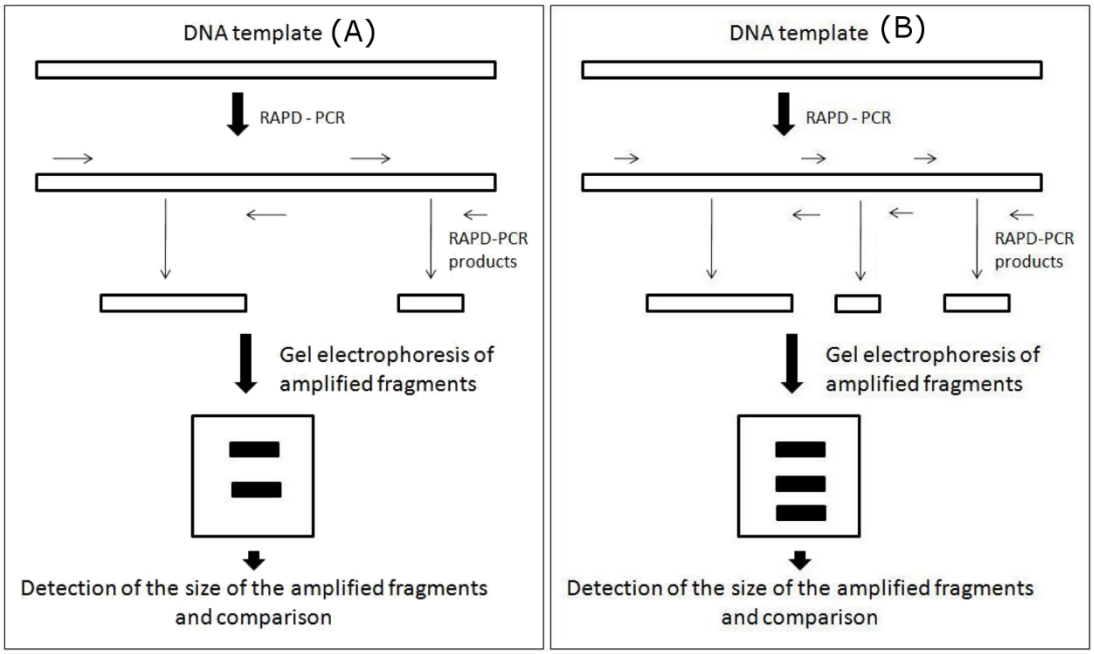

Figure 4.2 – The process of how random amplification of polymorphic DNA can result in different fingerprints for multiple DNA templates. In template A there was two products amplified fragments. However, in template B there was an additional amplified fragment as can be seen in gel electrophoresis meaning a different banding pattern is seen. This is indicative of genetic diversity between DNA template A and DNA template B. Figure adapted from Arif et al. 2010.

4.1.6 Aims and Hypotheses

In this chapter the aim was to develop a robust reproducible RAPD protocol using control isolates of *S. aureus* and *E. faecalis.* Using the developed protocol isolates were assessed for RAPD banding patterns followed by analysis using bionumerics software. To determine genotypic and phenotypic diversity, dendrograms based on RAPD profiles and antibiotic resistance were produced. The hypothesis was that RAPD and antibiotic resistance profile comparisons would show that there is genomic and phenotypic diversity.

4.2 Materials and Methods

4.2.1 DNA extraction

DNA was extracted for each biobank isolate for use in RAPD-PCR using the 'Chelex' method; a chelating agent used in combination with cell lysis (by heating) for high quality, high yield DNA extraction (HwangBo *et al.*, 2010). The protocol used was adapted from that described by HwangBo et al. (2010) who successfully assessed the Chelex method showing successful PCR using DNA extracted using Chelex 100. Similarly, Reyes-Escogido et al. (2010) compared a Chelex 100 based protocol with alternative methods and again demonstrated successful PCR using Chelex 100 extracted DNA. Beads (cryovials) for each isolate were recovered from -80 °C and aseptically streaked onto fresh MHA. This was incubated overnight at 37 °C for growth. The resulting biomass was re-suspended in 1 mL aliquots of ice cold PBS. This was centrifuged at 12,000 rpm for 10 min at 4 °C using an Eppendorf centrifuge 5418R (VWR, UK). Supernatant was decanted, the cell pellet re-suspended in 1 mL of fresh ice-cold PBS centrifuged at 12,000 rpm for 10 min at 4 °C and the step repeated twice. Finally, the supernatant was removed, and cell pellet re-suspended in 300 µL 5% w/v Chelex 100 (Sigma, UK) and incubated at 100 °C for 15 min on a Stuart block heater SBH200D (Stuart equipment, UK). Samples were then centrifuged at 12,000 rpm for 10 min at 4 °C and supernatant transferred to a clean Eppendorf in 100 µL aliquots and stored at -20 °C until use.

4.2.2 RAPD Primers

Primers used in RAPD are short arbitrary primers with no specific target gene. Both primers used are presented in Table 4.1. For random amplification of *S. aureus* isolates DNA primer 'AP-7' was used. This has previously been used for successful RAPD on both MSSA and MRSA

isolates (named RAPD-7 rather than AP-7) and for RAPD of *Klebsiella pneumonia* (Ashayeri-panah 2012). Primer 'R5' was used for RAPD of *Enterococcus* spp. isolates. This has previously been used for RAPD and showed high levels of discrimination compared to other RAPD primers (Martin *et al.*, 2005) and has recently been successfully used for RAPD of *E. faecalis* strains (Cheng *et al.*, 2017). Primers were purchased in lyophilised form (Invitrogen), and were re-constituted to a stock concentration of 100 μM using molecular grade water then diluted to a working concentration of 10 μM.

Table 4.1 – Primers used in Random Amplification of Polymorphic DNA of *Staphylococcus aureus* and *Enterococcus* spp. isolates.

Bacteria	**Primer**	**Primer Sequence (5'–3')**	**Reference**
S. aureus	AP-7	GTGGATGCGA	(Grinholc, Wegrzyn and Kurlenda, 2007)
Enterococcus spp.	R5	AACGCGCAAC	(Martin *et al.*, 2005)

4.2.3 RAPD-PCR Protocol

All reagents were purchased from Invitrogen unless otherwise stated and PCR reactions were carried out using a TC5000 PCR machine (Techne, UK). DNA extracted from isolates was subject to PCR in a final individual reaction volume of 25 μL; 24 μL RAPD PCR mastermix (Table 4.2) and 1 μL of genomic DNA were mixed in 0.2 mL PCR tubes (VWR). PCR was then completed using the cycle conditions summarised in Table 4.3. Each isolate was subject to RAPD-PCR (reaction mixture volume of 25 μL) in duplicate independent runs. Following PCR isolates were subject to gel electrophoresis.

Table 4.2 – PCR mastermix used for PCR reaction in RAPD analysis of *S. aureus* and *Enterococcus* spp. isolates.

PCR reagent	**Volume (for 34 reactions)**
PCR buffer (10 μM)	85 μL
dNTP mix (0.2 mmol)	68 μL
Primer (10 μM)	34 μL
Taq DNA polymerase	17 μL
$MgCl_2$ (50 mM)	68 μL
Molecular grade H_2O (Sigma)	578 μL
Total	**850 μL**

Table 4.3 – PCR program used for RAPD analysis of *S. aureus* and *Enterococcus* spp. isolates.

Temperature	**Time**	**Number of Cycles**
94 °C	5 min	-
94 °C	45 s	35
35 °C	1 min	
72 °C	2 min	
10 °C	Pause	-

4.2.4 Gel Electrophoresis

Following PCR, samples were subject to gel electrophoresis in a bio-rad gel electrophoresis tank using a PowerPac Basic battery (Bio-rad). 2% 12.5 cm agarose gels were prepared by dissolving (heating in microwave) 5 g of molecular biology grade agarose (Appleton, UK) in 250 mL 1X Tris/Borate/EDTA (TBE) buffer (Invitrogen), 25 µL of Sybr® Safe DNA gel stain (Invitrogen) was added then the gel allowed to set in the casting tray. Subsequently gels were placed in a Bio-rad gel tank and immersed in 1500 mL of 1X TBE buffer. A 10 µL aliquot of PCR product was mixed with 2 µL 6X DNA gel loading dye (Invitrogen) and 10 µL of this mixture was loaded into an individual well on the gel. 5 µL of a 100-base pair ladder was loaded into the first, middle and last lanes of each gel. Gels were then subject to electrophoresis at 100 V for 3h. Gels were visualised using G:BOX F3 gel doc system (Syngene Europe, UK) and each gel was imaged using the default settings on the Syngene software for standardisation purposes.

4.2.5 Validation of reproducibility of Gel Electrophoresis

A series of validation steps were undertaken to ensure reproducibility of the gel electrophoresis step, i.e. will the same PCR product run multiple times on one gel result in the same banding pattern (RAPD profile). For this control isolates *S. aureus* ATCC43300, *S. aureus* DSM20231, *E. faecalis* ATCC29212 and *E. faecalis* DSM12956 were used. The RAPD protocol (as described above) was used for RAPD assessment of these isolates followed by gel electrophoresis (as described above). One RAPD-PCR reaction was completed for each isolate, in gel electrophoresis steps, independent aliquots of the same PCR reaction were added to individual wells of the gel. The electrophoresis results were then assessed for reproducibility between replicates.

4.2.6 Validation of Intra-reproducibility of RAPD

Intra-reproducibility is the replication of an observation/measurement within a single experiment. In this case the intra-reproducibility is the replication of the RAPD profile of control isolates assessed in a single PCR reaction and on a single gel. However, multiple replications of the same isolate were tested. Therefore, this is a validation of the intra-reproducibility of a given isolate using the RAPD system designed; i.e. will the same isolate tested multiple times result in the same RAPD profile. The same procedures were completed as above, however the additional sample was included so reproducibility of the PCR could be assessed, i.e. two PCR runs of the same sample and subsequent processes.

4.2.7 Validation of Inter-reproducibility of RAPD

Inter-reproducibility is the replication of an observation/measurement between multiple independent experimental runs. In this case the inter-reproducibility is the replication of the RAPD profile of control isolates assessed in multiple independent PCR runs. This tests whether the same isolate tested in independent PCR runs of the RAPD protocol will result in the same RAPD profile, i.e. two separate PCR runs at different times.

For this control isolates *S. aureus* ATCC43300, *S. aureus* DSM20231, *E. faecalis* ATCC29212 and *E. faecalis* DSM12956 were used. The RAPD protocol (as described above) was used for RAPD assessment of these isolates followed by gel electrophoresis (as described above). The test described in *'Validation of Intra-reproducibility of RAPD'* above was repeated independent of that test and both results assessed for reproducibility.

4.2.8 Bionumerics Analysis of RAPD

Bionumerics is a software package commonly used for analysis of RAPD throughout the literature. Following validation of the RAPD protocol, each *S. aureus* and *Enterococcus* spp. uniform isolate was subject to RAPD analysis in duplicate independent PCR runs. To assess reproducibility of the two runs for each isolate, the band pattern was compared to each other for similarity using bionumerics software. Banding patterns were analysed using unweighted pair grouping method with arithmetic mean. Several comparison tests were then completed to produce similarity dendrograms. Each comparison was conducted with three reproducibility threshold parameters; i.e. [1] all isolates, [2] isolates which displayed >/=95% reproducibility and [3] isolates which displayed >/=99% reproducibility.

4.2.9 Bionumerics Analysis of Antibiotic Susceptibility Testing

Bionumerics software can be also used to compare antibiotic susceptibility antibiogram data. Each *S. aureus* and *Enterococcus* spp. isolate tested using EUCAST methods in *Chapter 3* resulted in an antibiogram profile for each isolate. These profiles can be compared to each other as a method of determining diversity between bacterial populations. Antibiogram data was input to the bionumerics software and comparisons conducted.

4.3 Results

4.3.1 RAPD Gel Output Example

The output of RAPD experiments used for analysis is a banding pattern visible under UV light following gel electrophoresis and staining with SybrSafe. In all RAPD assessments conducted duplicate gels were produced for each isolate and input to the bionumerics software for analysis. Two examples of gels produced using the described protocols are shown. Figure 4.3 shows a gel from validation/assessment of reproducibility steps conducted – the banding patterns seen across this gel are of the same isolate and can be seen to be consistent indicating reproducibility. Figure 4.4 shows the RAPD banding pattern for some of the hospital biobank isolates assessed – the differences in banding pattern between isolates is an indicator of genomic diversity. Such diversity of the biobank was subsequently assessed using the bionumerics software.

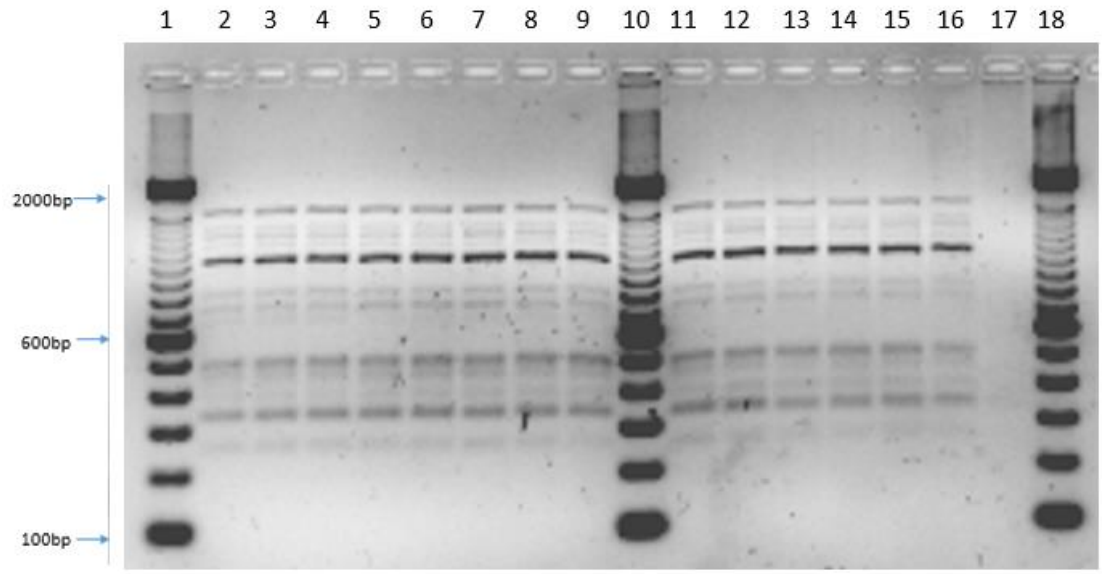

Figure 4.3 – RAPD gel showing analysis of reproducibility assessment of *Staphylococcus aureus* DSM20231. 2% agarose gel stained with SybrSafe imaged under UV light. Lanes 1 = 100bp ladder; lanes 2-6 = same PCR product of *S. aureus* DSM20232 RAPD (assessment of gel reproducibility); lanes 7-9 = replicates of *S. aureus* DSM20231 RAPD (assessment of RAPD reproducibility); lane 10 = 100bp ladder; lanes 11-16 replicates of *S. aureus* DSM20231 RAPD; lane 17 = negative no DNA control; lane 18 = 100bp ladder.

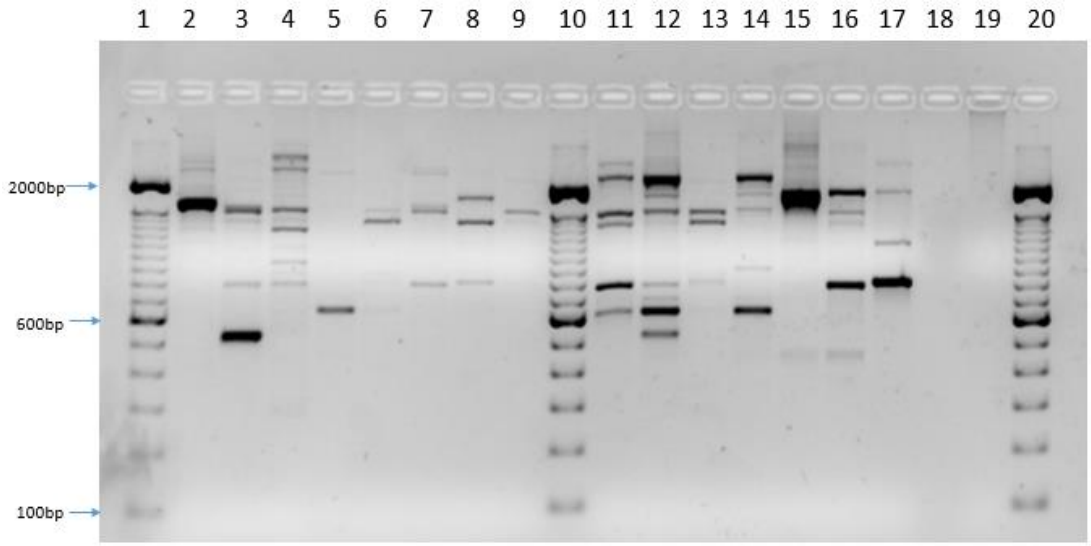

Figure 4.4 –RAPD gel showing isolates from the hospital uniform biobank. 2% agarose gel stained with SybrSafe imaged under UV light. Lane 1 = 100bp ladder; lane 2 = EA5; lane 3 = EA13; lane 4 = EA21; lane 5 = EA39; lane 6 = EA40; lane 7 = EA43; lane 8 = EA44; lane 9 = EA45; lane 10 = 100bp ladder; lane 11 = EA46; lane 12 = EA47; lane 13 = EA49; lane 14 = EA52; lane 15 = EA53; lane 16 = EA54; lane 17 = EA55; lane 18 = empty; lane 19 = negative no DNA control; lane 20 = 100bp ladder.

4.3.2 Assessment of Reproducibility of RAPD Protocol using Control Isolates

Due to the nature of RAPD assessment it is vital to ensure reproducibility of the protocol. Gel electrophoresis, intra-reproducibility and inter-reproducibility were all assessed and validated to ensure a robust RAPD method. Control isolates were used to assess reproducibility as outlined in materials and methods section. These assessments were conducted on the same gel (gel electrophoresis validation and intra-reproducibility check) then repeated independently to ensure reproducibility of the overall protocol (inter-reproducibility). Banding patterns from these gels were input to the bionumerics software and similarity comparisons conducted to quantitate reproducibility. Example dendrograms for *S. aureus* ATCC43300 and *E. faecalis* ATCC29212 are shown in Figure 4.5 and Figure 4.6; also shown is a summary Table 4.4 detailing the results (% similarity) for various reproducibility tests completed for all control isolates.

The dendrogram for the *S. aureus* ATCC43300 control isolate clearly highlights good reproducibility in the RAPD protocol. Two major clades can be seen with 89.9% similarity (which represents similarity as it is replicates of the same sample) to each other – these clades represent the inter-reproducibility between RAPD profiles for replicate and replicate 2 of the protocol for this isolate. Intra- reproducibility can also be seen within these major clades (two clades represent two independent PCR runs for the same smaple); within these independent runs there is >95% similarilty. The *E. faecalis* ATCC29212 dendrogram also demonstrates reproducibility with inter-reproducibility being ~95%.

Figure 4.5 – Dendrogram showing similarity (%) in RAPD profiles of reproducibility assessment for *S. aureus* ATCC43300. Key: • = replicate 1; * = replicate 2; independent PCR runs denoted a-j; repeats of a further sub-categorised a1-a5.

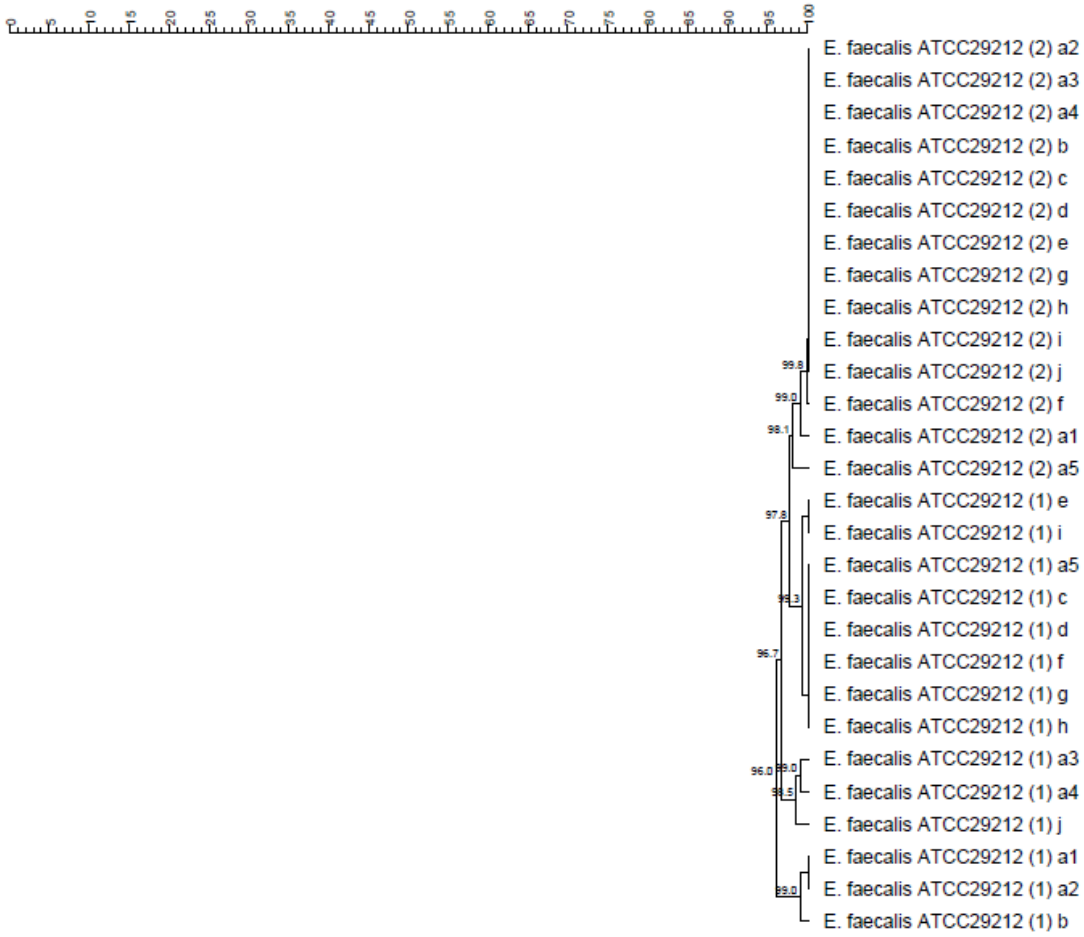

Figure 4.6 – Dendrogram showing similarity (%) in RAPD profiles of reproducibility assessment for *E. faecalis* ATTCC29212. Key: (1) = replicate 1; (2) = replicate 2; independent PCR runs denoted a-j; repeats of a further sub-categorised a1-a5.

Table 4.4 – Summary of reproducibility assessment of RAPD protocol.

	S. aureus **ATCC43300**	***S. aureus*** **DSM20231**	***E. faecalis*** **ATCC23212**	***E. faecalis*** **DSM12956**
Gel electrophoresis reproducibility	98.3%	97.5%	98.1%	98.8%
Intra-reproducibility	97.9%	92.8%	98.1%	98.8%
Inter-reproducibility	89.9%	86.2%	96%	97.8%

Numbers are representative of the lowest similarity value for each test. Gel electrophoresis is similarity between isolates denoted a1-a5 in example dendrograms. Intra-reproducibility is similarity between one full RAPD run, i.e. denoted (1)a-j or (2)a-j in example dendrograms. Inter-reproducibility is the lowest similarity value in a comparison of (1) vs (2).

4.3.3 Assessment of Reproducibility of RAPD analysis of Uniform Isolates

Once the protocol was optimised, validated and assessed for reproducibility (as descibed in section 4.3.2) analysis of *S. aureus* and *Enterococcus* spp. isolates from post-shift healthcare workers uniforms was undertaken. As the assessment of reproducibility with the control isolates highlighted variation in the results – RAPD for each biobank isolate was performed in duplicate in two independent RAPD runs of the described protocol. The two resultant banding patterns for each isolate were then assessed for reproducibility. Figure 4.7 shows an example of a dendrogram produced for one isolate comparing independent experiment 1 and indpendant experiment 2 (of the same isolate). This shows how the similarity values were determined by an independent assessment of reproducibility using the Bionumerics software to produce a dendrogram for each isolate. Table 4.5 shows the reproducibility similarity value between replicate 1 and replicate 2 of each isolate.

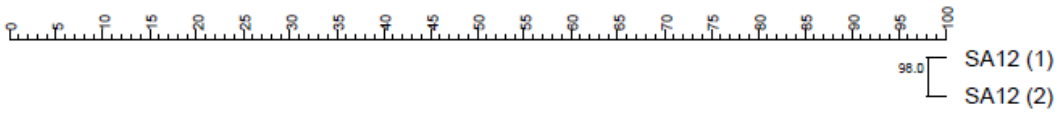

Figure 4.7 – Example of dendrogram produced to assess similarity (%) between replicate 1 and replicate 2 of a isolates SA12 (replication of full RAPD protocol).

Table 4.5 – Reproducibility analysis of each isolates assessed using RAPD. Table shows percentage similarity between independent replicates of RAPD for each isolate.

Staphylococcus aureus						***Enterococcus* spp.**					
Isolate	Similarity (%)	Isolate	Similarity (%)	Isolate	Similarity (%)	**Isolate**	**Similarity (%)**	**Isolate**	**Similarity (%)**	**Isolate**	**Similarity (%)**
SA1	95	SP1	99	SN1	96	EA1	97	EP1	97	EN1	99
SA10	94	SP2	99	SN4	92	EA2	91	EP3	98	EN2	99
SA11	93	SP3	99	SN5	94	EA3	94	EP4	99	EN3	99
SA12	98	SP5	99	SN7	97	EA6	98	EP5	98	EN4	99
SA13	97	SP6	96	SN8	97	EA7	97	EP6	98	EN5	100
SA14	96	SP7	98	SN10	97	EA10	97	EP7	98	EN6	100
SA15	95	SP8	99	SN11	96	EA11	97	EP8	100	EN9	99
SA16	93	SP9	99	SN12	96	EA14	99	EP9	99	EN12	93
SA17	92	SP10	94	SN14	93	EA16	95	EP13	100	EN13	98
SA21	96	SP11	93	SN15	94	EA20	97	EP15	99	EN15	100
SA22	100	SP12	98	SN16	95	EA22	98	EP16	98	EN16	99
SA24	100	SP14	98	SN17	95	EA23	98	EP17	97	EN17	97
SA25	97	SP15	90	SN20	97	EA24	99	EP18	100	EN22	100
SA26	98	SP16	94	SN21	95	EA25	98	EP21	100	EN24	99
SA27	98	SP18	94	SN23	97	EA26	98	EP24	98	EN29	93
SA28	96	SP20	94	SN31	94	EA27	98	EP26	96	EN31	99
SA29	92	SP22	98	SN32	87	EA28	98	EP27	98	EN32	98
SA34	99	SP23	96	SN34	92	EA29	98	EP28	99	EN33	98
SA36	96	SP25	94	SN35	97	EA30	98	EP30	99	EN36	98
SA37	99	SP26	96	SN37	99	EA31	98	EP31	98	EN39	97
SA38	98	SP29	95	SN38	96	EA32	98	EP32	98	EN41	97
SA39	99	SP30	98	SN39	98	EA33	96	EP34	96	EN42	97
SA40	99	SP32	99	SN40	89	EA34	98	EP2	99	EN45	99
SA41	99	SP33	98	SN41	92	EA36	98	EP23	98	EN46	99
SA42	98	SP34	98	SN42	98	EA37	93	EP59	96	EN47	100
SA43	99	SP35	99	SN43	92	EA41	98	EP60	95	EN48	99
SA44	99	SP36	99	SN44	92	EA42	98			EN49	100
SA45	97	SP37	99	SN22	98	EA5	96			EN50	100
SA46	97	SP38	99	SN24	97	EA13	95			EN51	100
SA47	99	SP39	95	SN26	97	EA21	96			EN52	100
SA48	94	SP41	98	SN27	94	EA39	99			EN53	99
SA49	97	SP42	99	SN46	92	EA40	99			EN54	100
SA50	99	SP43	97	SN47	94	EA43	97			EN55	100
SA51	98	SP44	98	SN48	93	EA44	98			EN56	100
SA52	97	SP45	98	SN49	95	EA45	99			EN57	100
SA57	95	SP46	97	SN50	93	EA46	94			EN58	99
SA58	92	SP47	93	SN51	98	EA47	93			EN59	100

SA59	96	SP48	98	SN52	97	EA49	97			EN63	100
SA62	96	SP49	94	SN53	93	EA52	95			EN64	97
SA64	94	SP50	99	SN54	97	EA53	98			EN69	98
SA65	97	SP51	95	SN55	98	EA54	97			EN71	98
SA66	95	SP52	93	SN57	92	EA55	97			EN73	99
SA67	93	SP53	96	SN58	96					EN76	99
SA68	94	SP57	98	SN59	94					EN80	99
SA69	98	SP58	99	SN60	95					EN81	99
SA70	97	SP60	97	SN61	98					EN82	99
SA71	98	SP64	97	SN62	95						
SA72	95	SP65	99	SN63	96						
SA74	98	SP66	96	SN64	90						
SA79	97	SP69	96	SN65	90						
SA80	99	SP70	89	SN66	93						
SA84	98	SP71	99	SN67	93						
SA87	99	SP73	92	SN68	96						
SA88	98	SP74	100	SN69	89						
SA89	99	SP75	95	SN70	99						
SA91	98	SP76	97	SN72	95						
SA93	98	SP78	97	SN74	96						
SA94	98	SP79	92	SN76	98						
SA95	99	SP81	100	SN78	99						
SA96	97	SP82	95	SN79	96						
SA97	96	SP84	92	SN80	96						
SA98	94	SP85	89	SN81	96						
SA99	99	SP86	93	SN82	98						
		SP88	93	SN83	93						
		SP89	98	SN86	99						
		SP90	96	SN88	96						
		SP91	94	SN89	95						
		SP93	97	SN90	95						
		SP95	94	SN91	96						
		SP96	90	SN92	95						
		SP97	94	SN93	95						
		SP98	93	SN94	98						
				SN97	99						
				SN99	98						
				SN100	96						

Similarity (%) values representative of similarity between two independent RAPD runs for each isolate. Bionumerics was used to determine the similarity between rep 1 and rep 2. Not all isolates were recoverable from -80°C hence variable sample sizes for each sample group.

For all *S. aureus* isolates three dendrogram figures were produced, [1] all *S. aureus* isolates; [2] *S. aureus* isolates with 95% or higher threshold for reproducibility between replicates and [3] *S. aureus* isolates with 99% or higher threshold for reproducibility between replicates. These dendrograms are shown in Figure 4.8, Figure 4.9 and Figure 4.10 respectively . The dendrogram displaying no reproducibility threshold (Figure 4.8) shows the most isolates and consequently apparent increased diversity amongst the population. However, the lowest reproducibility between replicates observed was 87% (Table 4.5). This therefore represents the point where any diversity shown between 87% up to 100% could in theory be a result of experimental irreproducibility, rather than genuine genomic diversity between isolates. When a 95% reproducibility threshold was included any diversity between 95% and 100% cannot be considered genomic diversity; however as reproducibility was directly assessed any diversity represented below 95% should be considered genomic diversity within the population; this diversity is observed in the dendrogram with some isolates showing ~91% similarity to each other.

The same can be noted when a 99% threshold was included; however this threshold represents a higher level of scrutiny of experimental reproducibility resulting in a more robust dataset as any genomic diversity observed (i.e. <99% similarity) should be considered representative of genomic diversity as testing has been conducted in a robust methodical manner and data has been assessed and validated for quality and reproducibility. This analysis set demonstrated as low as ~94% genomic similarity between some isolates. The caveat of including these parameters is that the sample size is reduced meaning the observation of any genomic diversity is limited. However as the aim of this work was to determine, as accuratley as possible, genomic diversity; subsequent analyses shown used a reproducibility threshold of 99%.

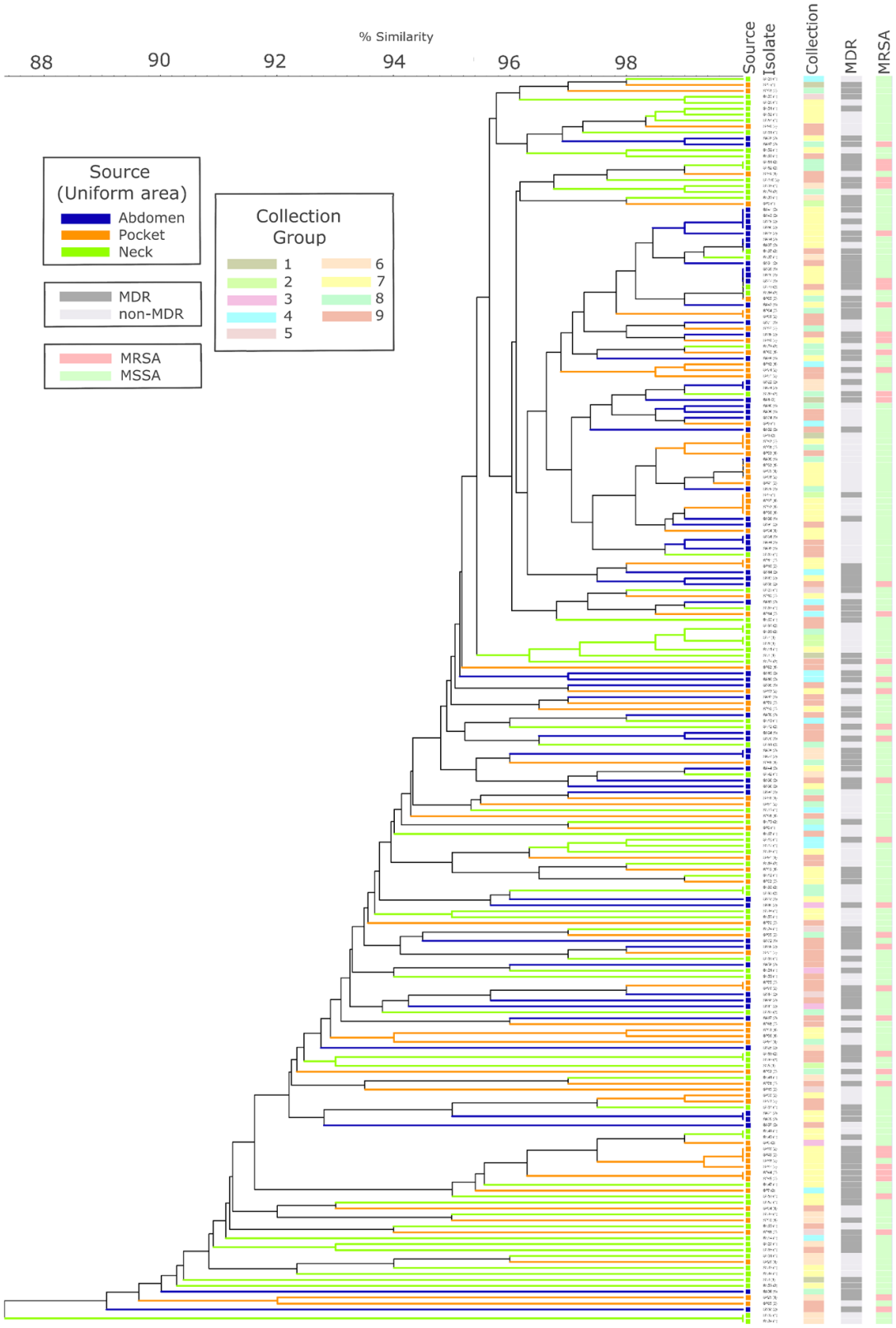

Figure 4.8 – Dendrogram including all *S. aureus* tested using RAPD with no reproducibility threshold. Dendrogram represenative of similarity (%) amongst bacterial population.

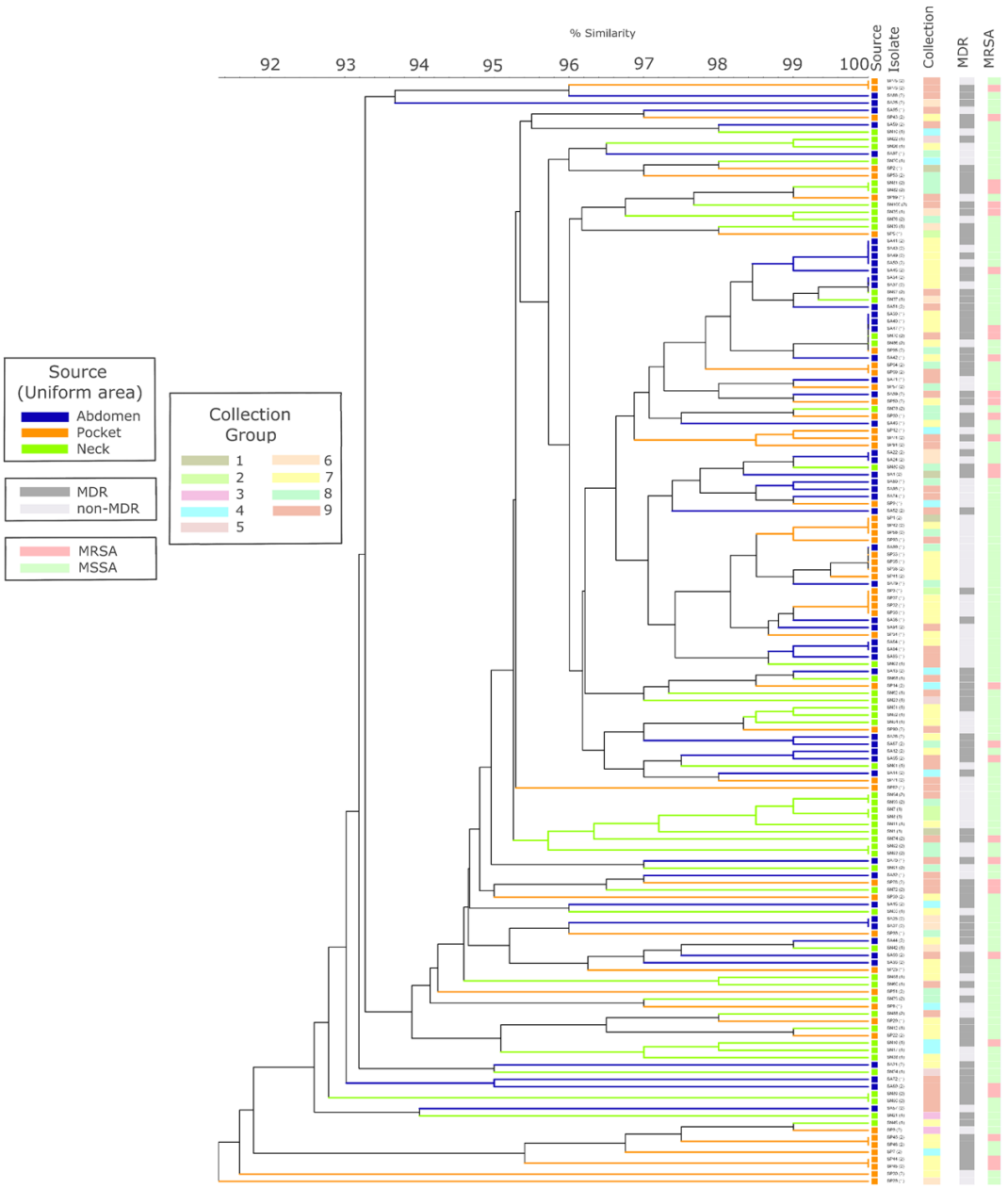

Figure 4.9 – Dendrogram including *S. aureus* isolates tested using RAPD which had a 95% or higher reproducibility value between experimental replicates. Dendrogram represenative of similarity (%) amongst bacterial population.

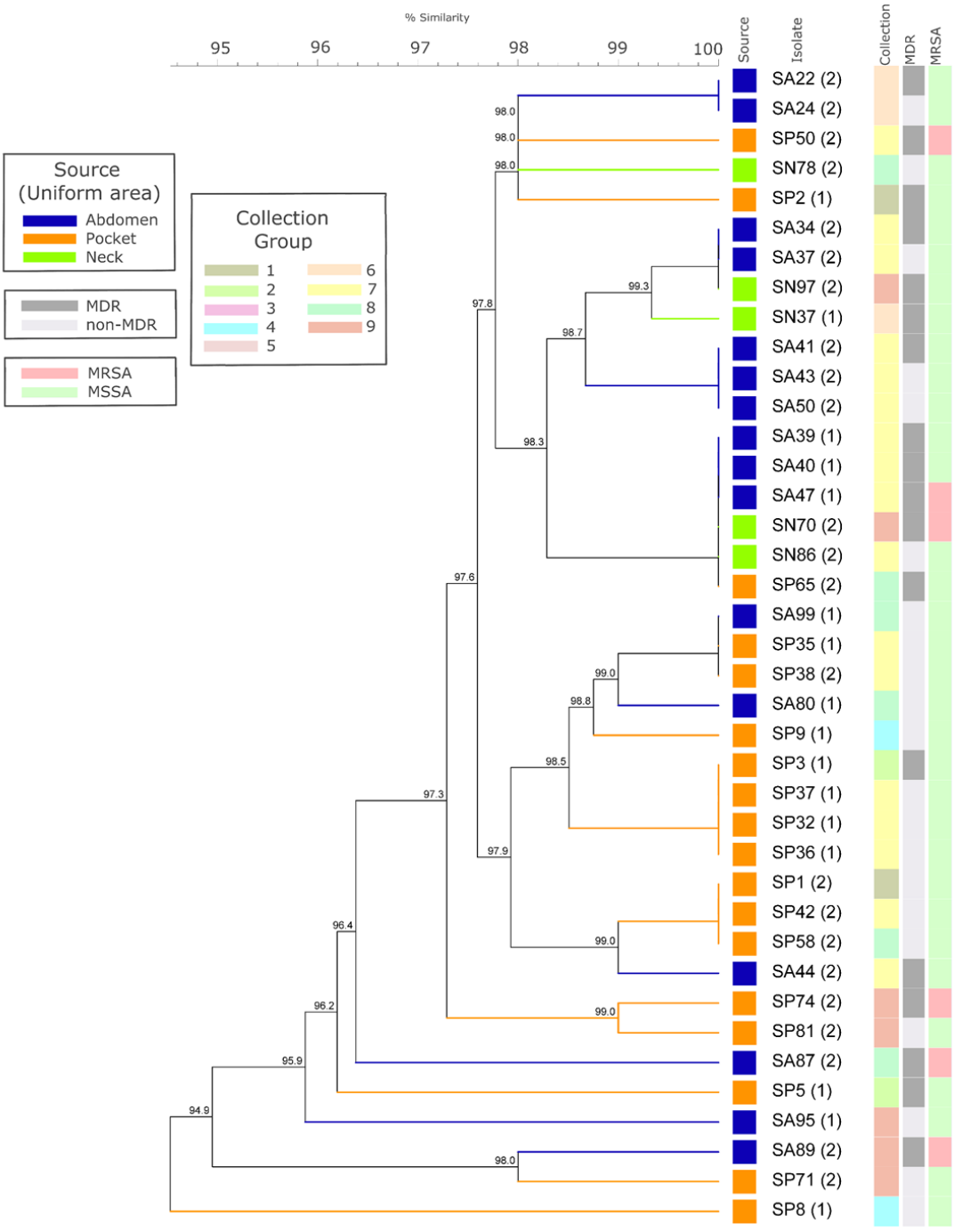

Figure 4.10 – Dendrogram including all *S. aureus* isolates tested using RAPD which had a 99% or higher reproducibility value between experimental replicates. Dendrogram represenative of similarity (%) amongst bacterial population.

4.3.4 *Staphylococcus aureus* Genomic diversity based on Antibiotic Profiling

Similarity analyses were conducted of the RAPD banding patterns of *S. aureus* isolates classed MRSA and MSSA by EUCAST assessment of isolates as described in chapter 3. Figure 4.11, Figure 4.12, Figure 4.13 and Figure 4.14 show the similarity dendrograms for MRSA and MSSA, MDR and non-MDR respectively. All *S. aureus* isolates (MRSA and MSSA) showed a minimum similarity of ~94%. Only 2 of 6 isolates MRSA isolates showed 100% similarity to one another; however one isolate showed <95% similarity to all other MRSA isolates. Interestingly all MRSA isolates were also MDR. Isolates deemed MSSA showed more clades with high levels of genomic similarity to each other. The minimum similarity seen was ~94%. Throughout the dendrogram small clusters with high similarity to each other can be seen, often these clusters arise from the same collection day and/or MDR classification. The *S. aureus* MDR isolates dendrogram shows a large clade of 14/18 isolates with 98% genomic similarity to each other, the other 4 isolates have decreasing similarity to as low as 94%. The non-MDR isolates showed a high percentage of similarity to each other with a 18/21 exhibiting 97.8% similarity to each other. Interestingly, all non-MDR are also MSSA and both non-MDR and MSSA isolates have minimum similarity of ~94%.

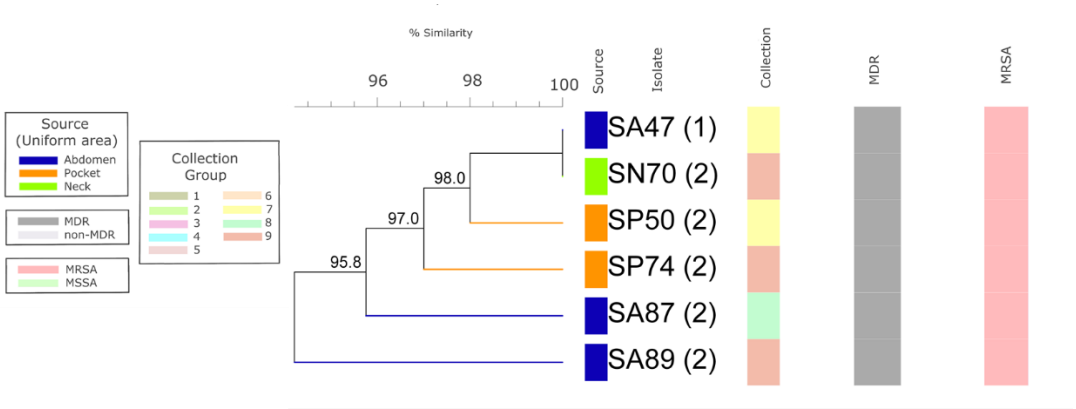

Figure 4.11 – Similarity dendrogram of MRSA *S. aureus* isolates recovered from post-shift healthcare workers uniforms. Isolates were classed MRSA by EUCAST disc diffusion testing. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

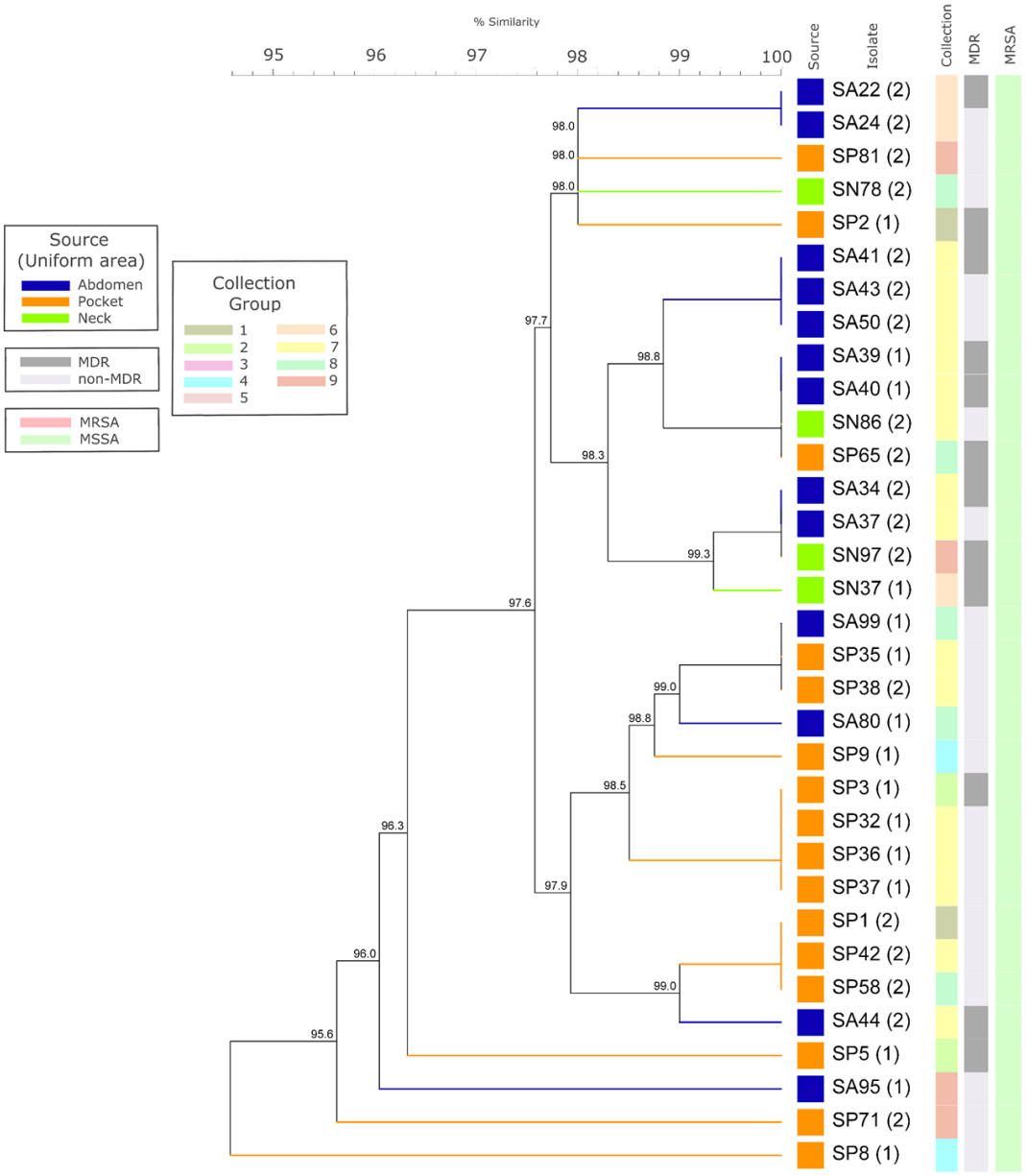

Figure 4.12 – Similarity dendrogram of MSSA *S. aureus* isolates recovered from post-shift healthcare workers uniforms. Isolates were classed MSSA by EUCAST disc diffusion testing. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

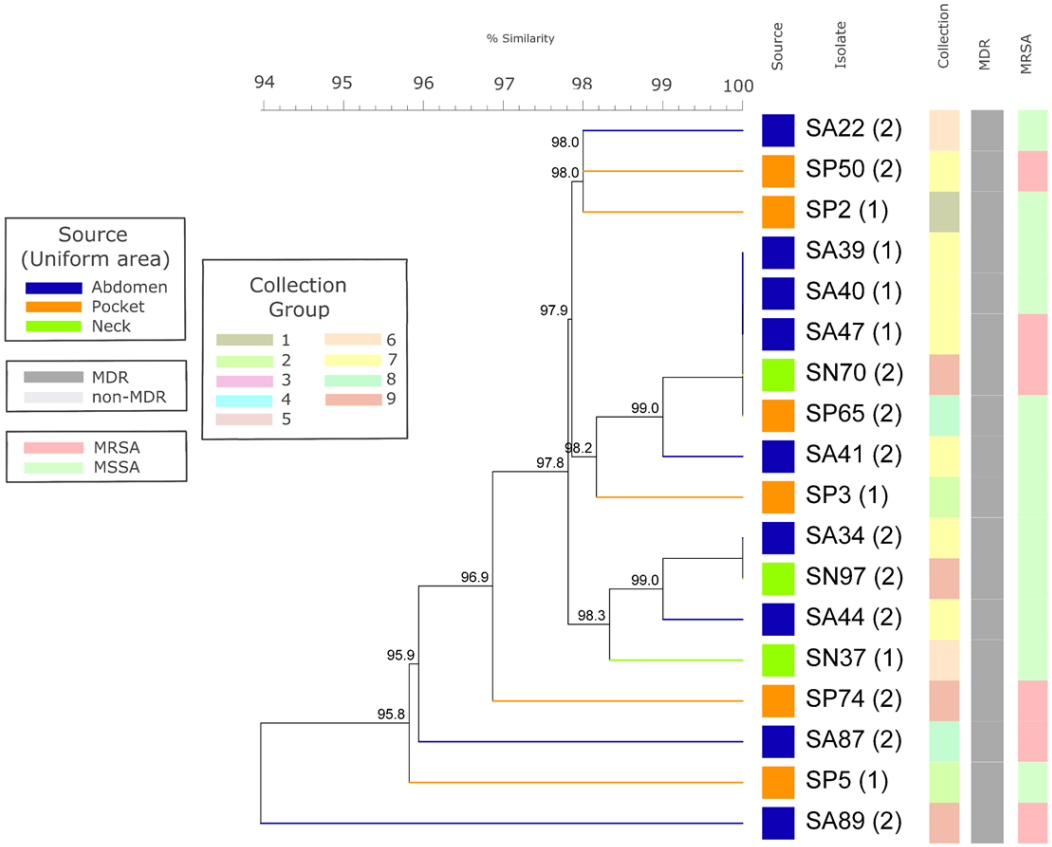

Figure 4.13 – Similarity dendrogram of MDR *S. aureus* isolates recovered from post-shift healthcare workers uniforms. Isolates were classed MDR by EUCAST disc diffusion testing. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

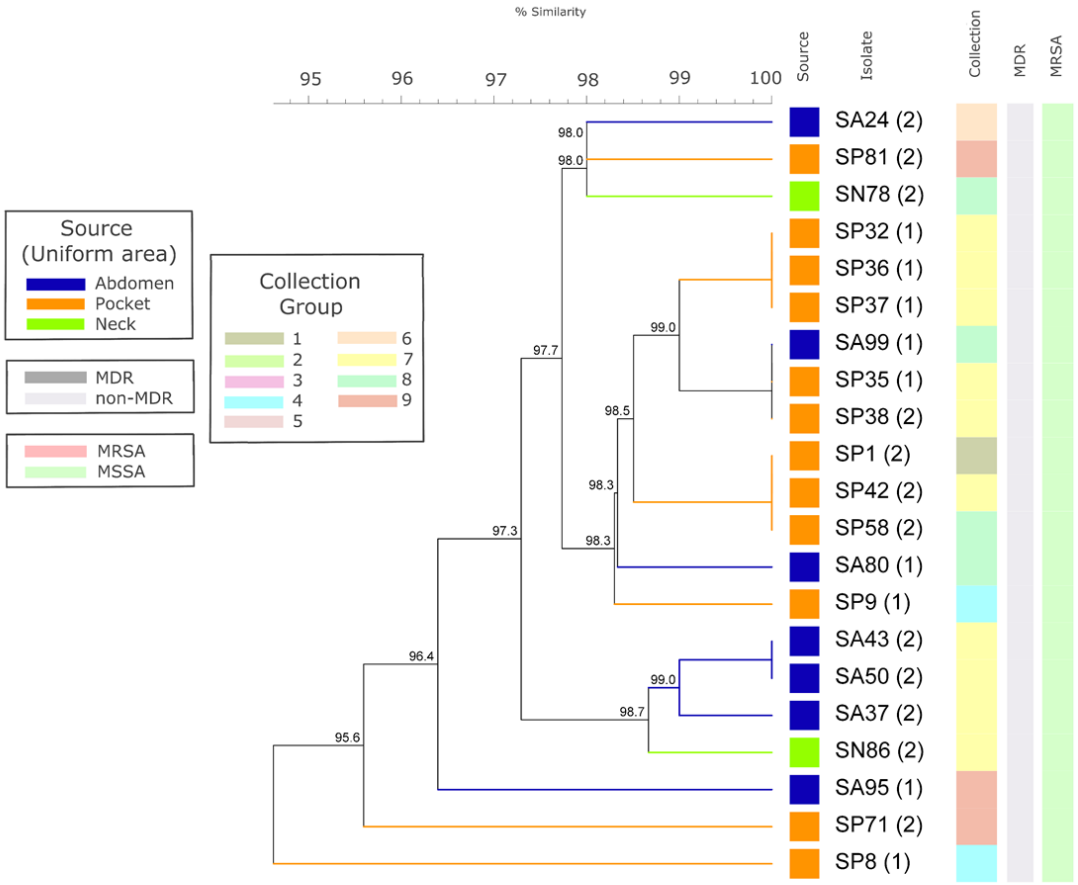

Figure 4.14 – Similarity dendrogram of non-MDR *S. aureus* isolates recovered from post-shift healthcare workers uniforms. Isolates were classed non-MDR by EUCAST disc diffusion testing. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

4.3.5 *Staphylococcus aureus* Genomic diversity based on Source of Isolation

S. aureus isolates assessed for genomic diversity using RAPD were isolated from post-shift healthcare workers uniforms at three locations (abdomen, pocket and neckline). Figure 4.15, Figure 4.16 and Figure 4.17 show the dendrograms for *S. aureus* abdomen, neckline and pocket isolates respectively.

Within the *S. aureus* population recovered from abdomen area of uniforms there is high similarity amongst all isolates (~94-100% similarity). However some isolates demonstrate increased similarity with isolates which have the same antibiotic profiling traits as each other, i.e. there are clades of MRSA, MSSA, MDR and non-MDR isolates with very high (99-100%) similarity values. A small number (5) of neck isolates could be included in this analysis and they all demonstrate 98-100% similarity – a larger population of isolates would be required to robustly determine diversity. Pocket isolates also display high similarity (lowest =95%) however, there are sub-populations present – within these sub-populations similar antibiotic profiles can be seen similar to that reported for the abdomen population.

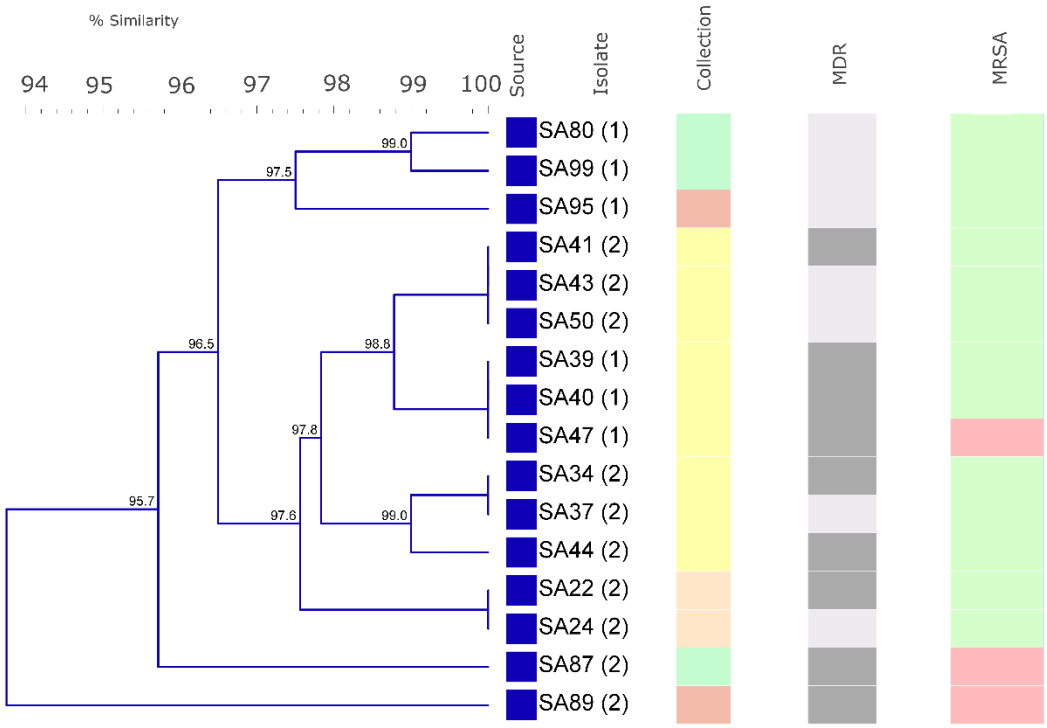

Figure 4.15 – Similarity dendrogram of *S. aureus* isolates recovered from abdomen of post-shift healthcare workers uniforms. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

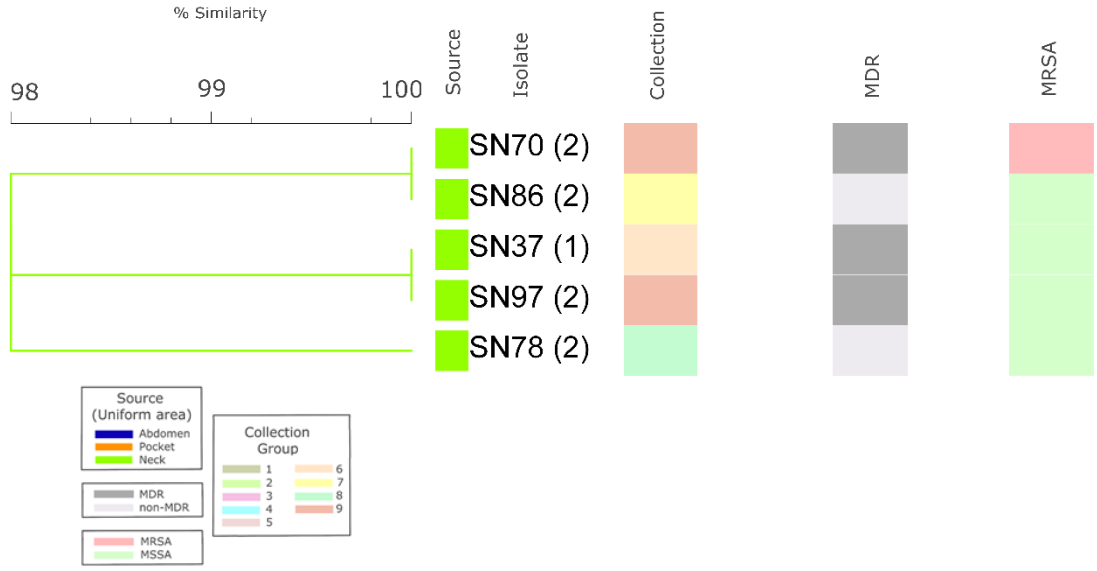

Figure 4.16 – Similarity dendrogram of *S. aureus* isolates recovered from neck of post-shift healthcare workers uniforms. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

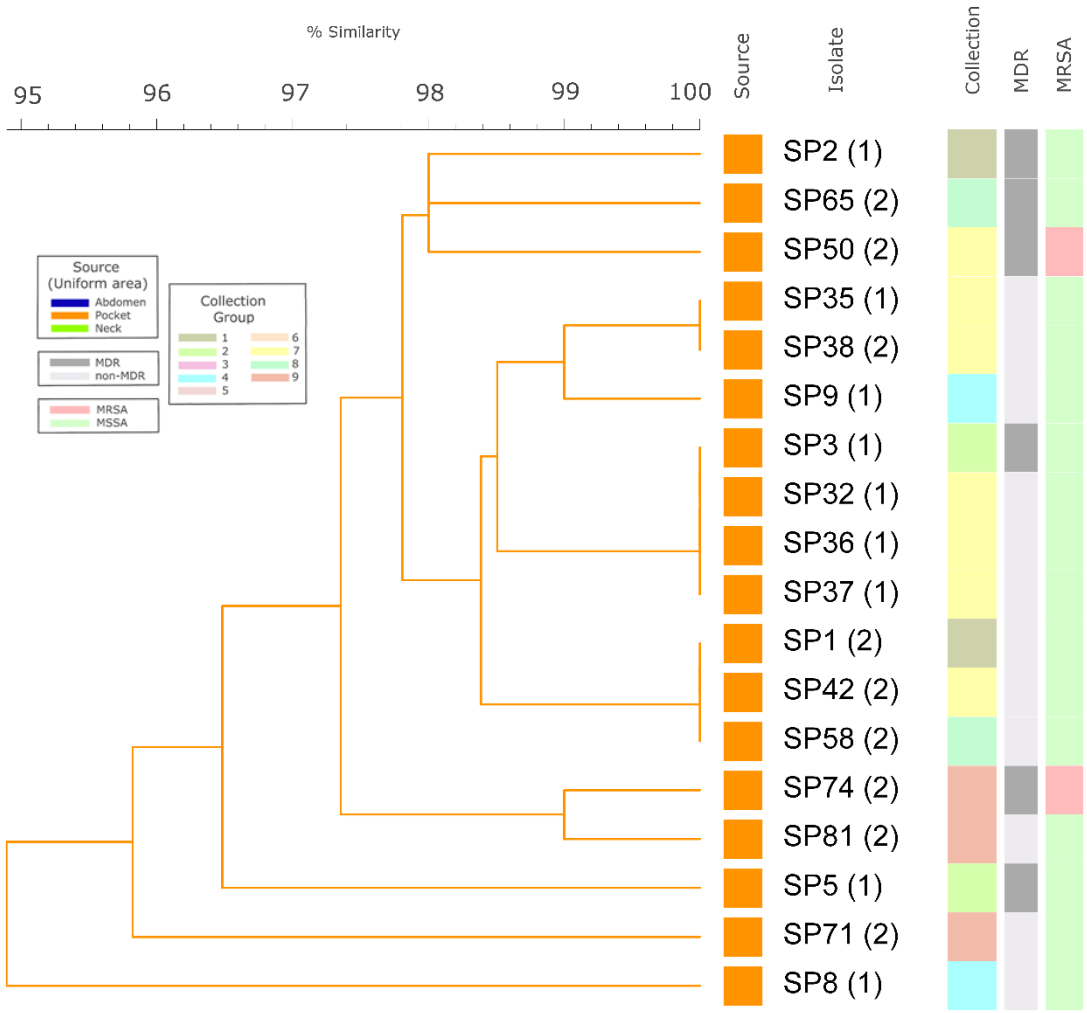

Figure 4.17 – Similarity dendrogram of *S. aureus* isolates recovered from pocket of post-shift healthcare workers uniforms. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

4.3.6 *Enterococcus* spp. Genomic diversity

A similarity test was conducted comparing RAPD banding patterns of all *Enterococcus* spp. isolates recovered from healthcare workers post shift uniforms (Figue 4.18). As with *S. aureus* analysis only isolates that demonstrated 99% or above reproducibility values between independent RAPD runs were included.

All *Enterococcus* spp. isolates show similarity as low as <93% with numerous sub-populations evident. Some of these sub-populations have similar antibiotic susceptibility patterns with clades of VRE or non-VRE isolates showing increased similarity. A small family of 9 isolates show the same characteristics (all VRE and same collection point) and demonstrate 100% similarity. Conversely there are families of non-VRE isolates which also display 100% similarity. Trends can also be seen regarding source of isolation specifically with *Enterococcus* spp. isolated from the neck having increased similarity compared to those isolates from abdomen or pocket areas.

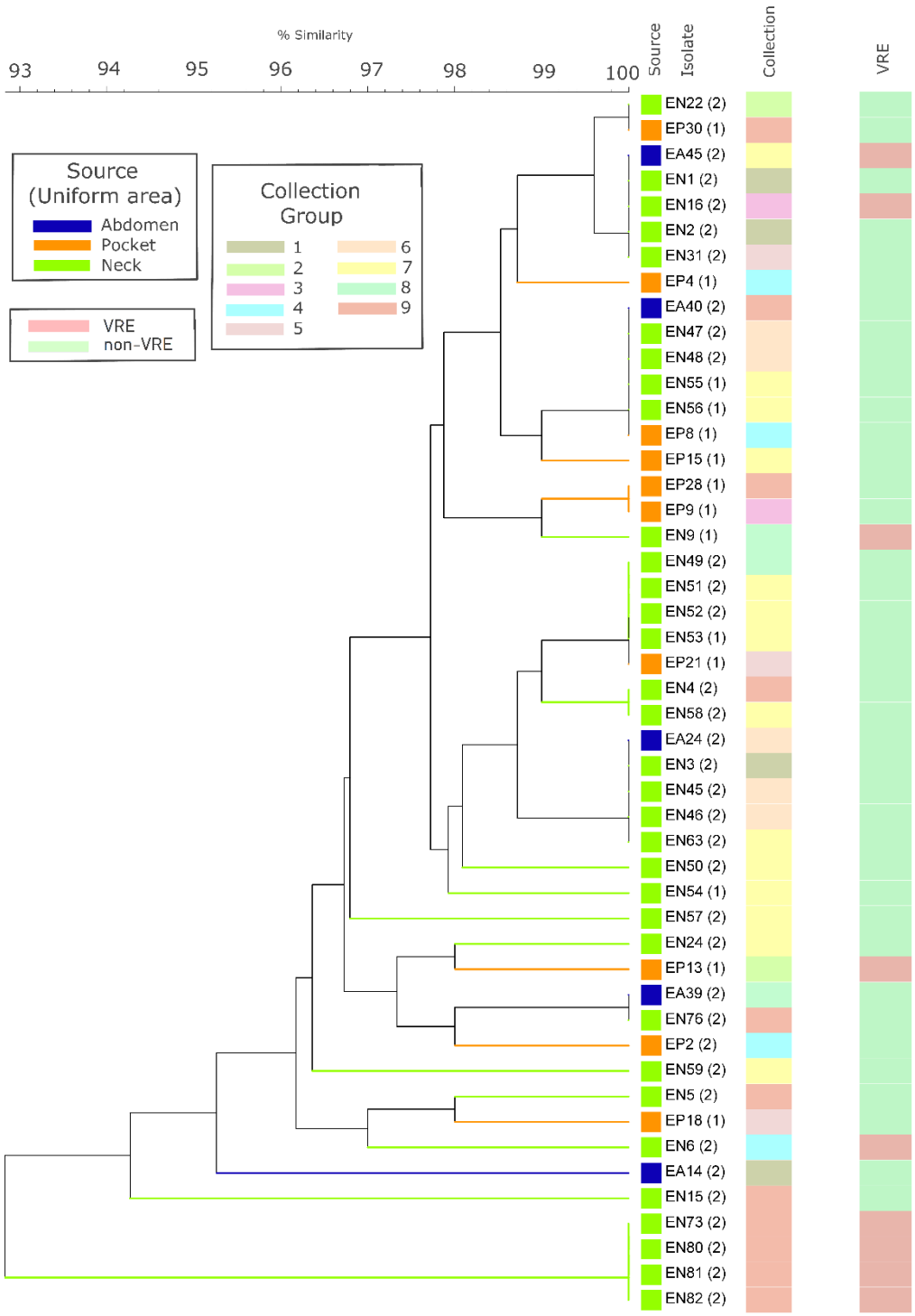

Figure 4.18 – Similarity dendrogram of *Enterococcus* spp. isolates recovered from post-shift healthcare workers uniforms. Similarity determined by RAPD testing and subsequent analysis using Bionumerics software (n=48). For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibility of 99% and above (between two independent replicates) were included in analysis.

4.3.7 *Enterococcus* spp. Genomic diversity based on Antibiotic Profiling

Similarity tests were conducted on *Enterococcus* spp. isolates recovered from healthcare workers uniforms instructing the software to use parameters based on antibiotic profiles determined by EUCAST (*chapter 3*). Similarity tests was conducted for all isolates deemed VRE and non-VRE, dendrograms shown in Figure 4.19 and Figure 4.20 respectively.

The VRE isolates dendrogram shows two distinctive clades with 94% similarity between the two clades. Within these clades there is high levels of similarity, one of them shows 100% similarity between four isolates – interestingly these four isolates arise from the same source (neck), same collection group (9) and are all classed VRE – these four isolates were recovered from two independent uniforms. The non-VRE isolates show increased diversity however, there is also an increased number of isolates. There is as low as 94% similarity and a range of sub-populations present with obvious trends where source of isolation shows increased similarity in certain clades.

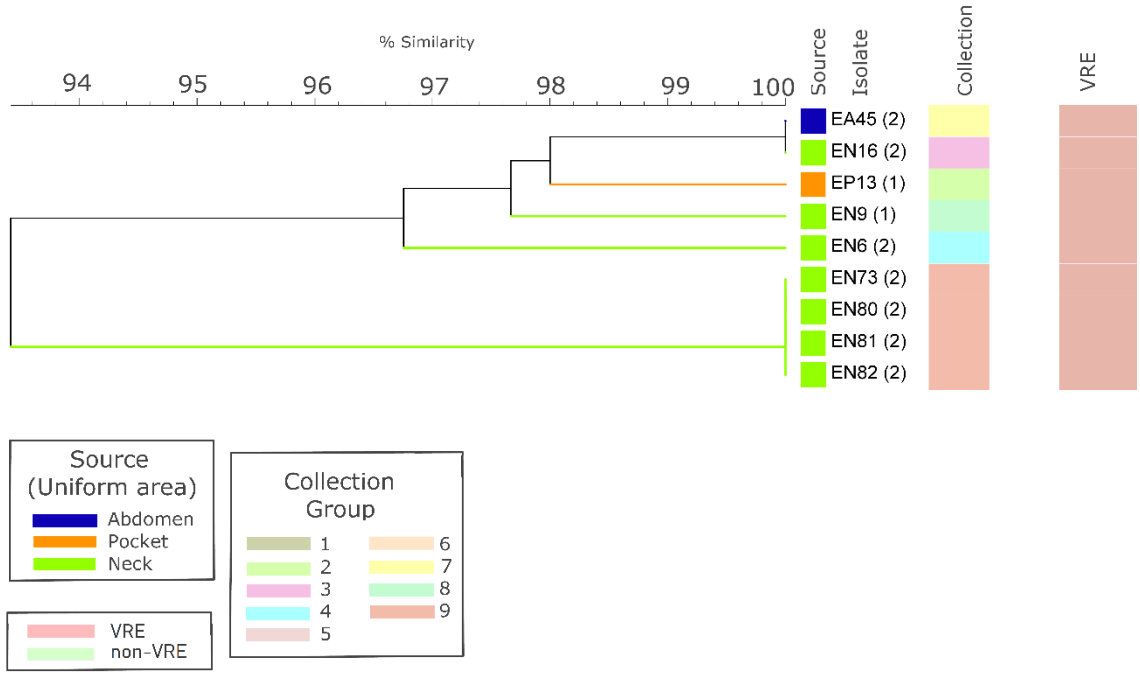

Figure 4.19 – Similarity dendrogram of VRE *Enterococcus* spp. isolates recovered from post-shift healthcare workers uniforms. Isolates were classed VRE by EUCAST disc diffusion testing. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

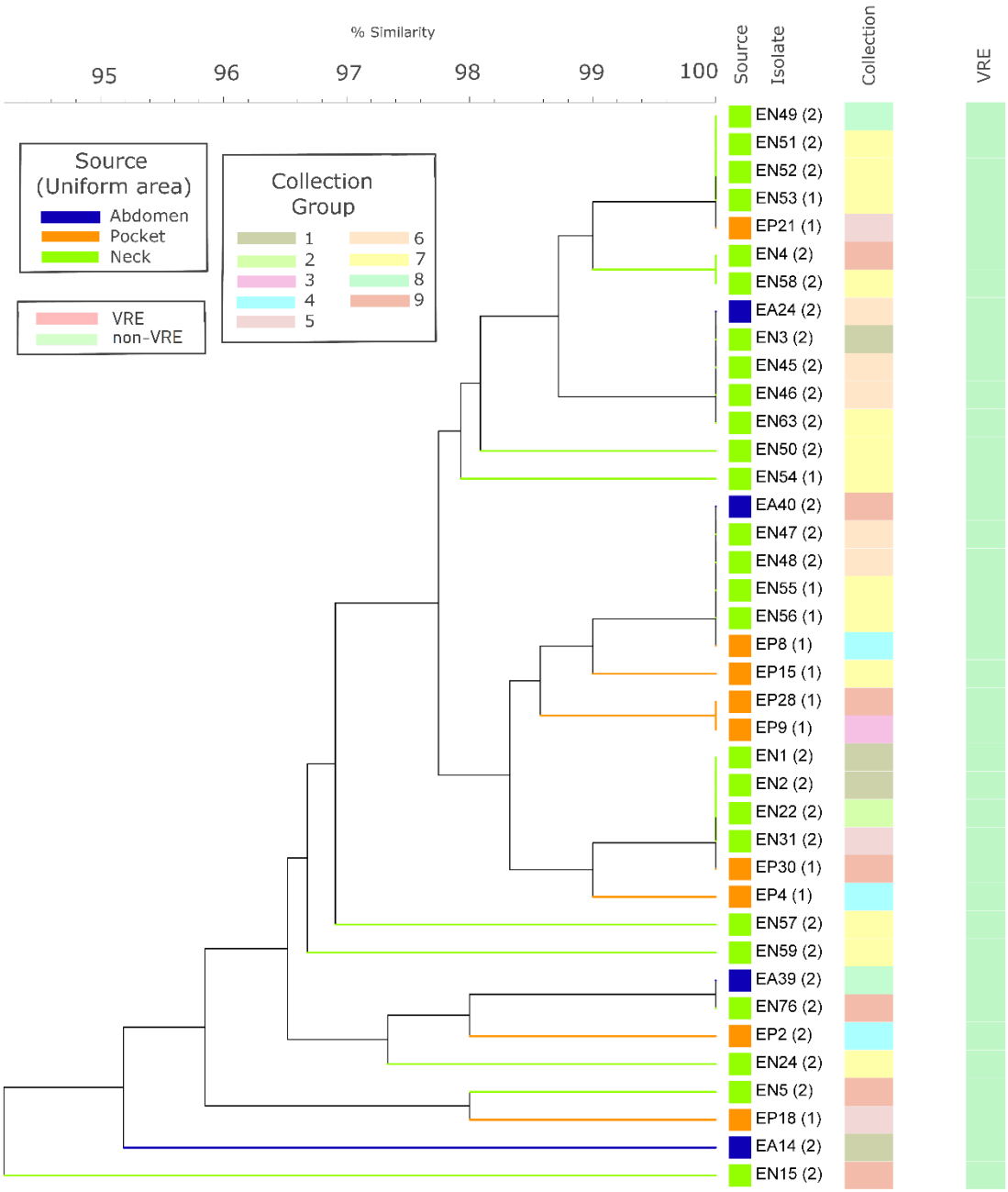

Figure 4.20 – Similarity dendrogram of non-VRE *Enterococcus* spp. isolates recovered from post-shift healthcare workers uniforms. Isolates were classed non-VRE by EUCAST disc diffusion testing. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

4.3.8 Enterococcus spp. Genomic diversity based on Source of Isolation

Similarity tests were conducted to assess diversity of *Enterococcus* spp. isolates based on the area of the uniform they were isolated from. Figure 4.21, Figure 4.22 and Figure 4.23 show dendrograms for similarity tests for abdomen, neck and pocket isolates respectively.

Only five isolates could be included in the similarity test for *Enterococcus* spp. isolates recovered from the abdomen site, therefore limited diversity is seen as a larger population would be required; however interestingly none of the isolates demonstrate 100% similarity to each other. Isolates recovered from the neck show increased diversity with lowest recorded similarity being ~93%, within the neck population VRE and non-VRE showed increased similarity with obvious clades/families of isolate present. There is also increased similarity to isolates recovered from in the same collection group. As noted with abdomen isolates no pocket isolates were 100% similar to each other.

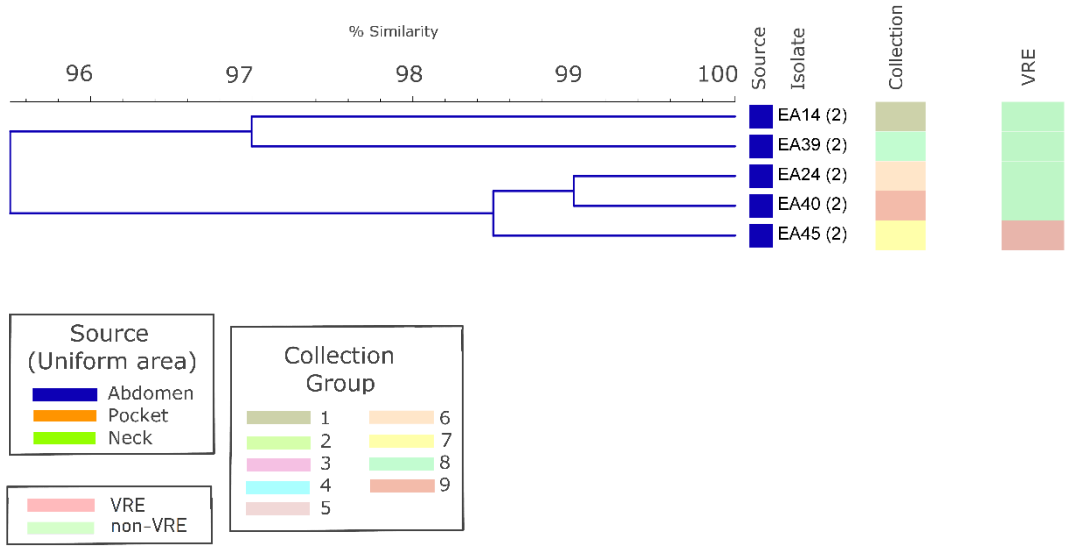

Figure 4.21 – Similarity dendrogram of *Enterococcus* spp. isolates recovered from abdomen of post-shift healthcare workers uniforms. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

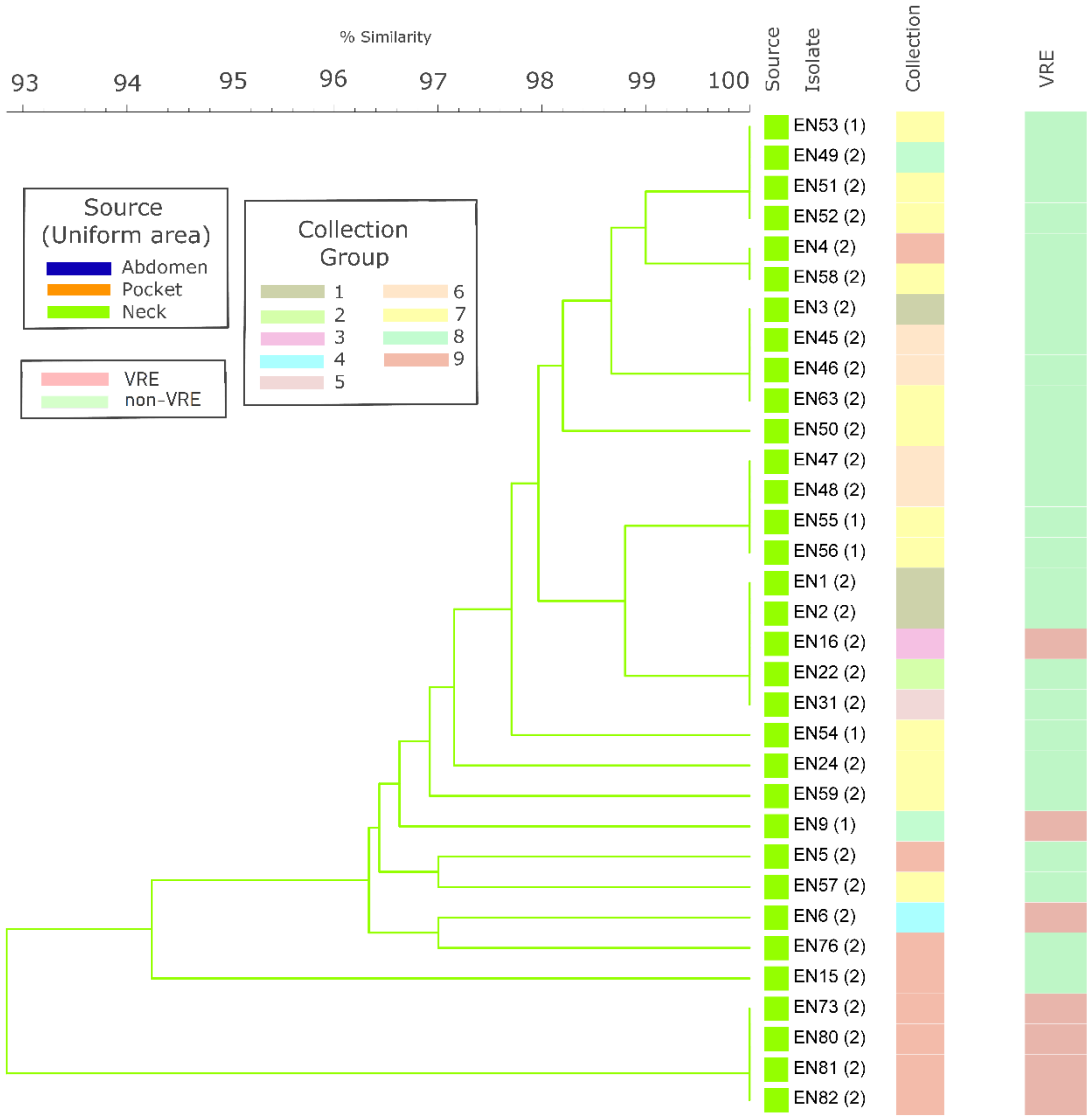

Figure 4.22 – Similarity dendrogram of *Enterococcus* spp. isolates recovered from neck of post-shift healthcare workers uniforms. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

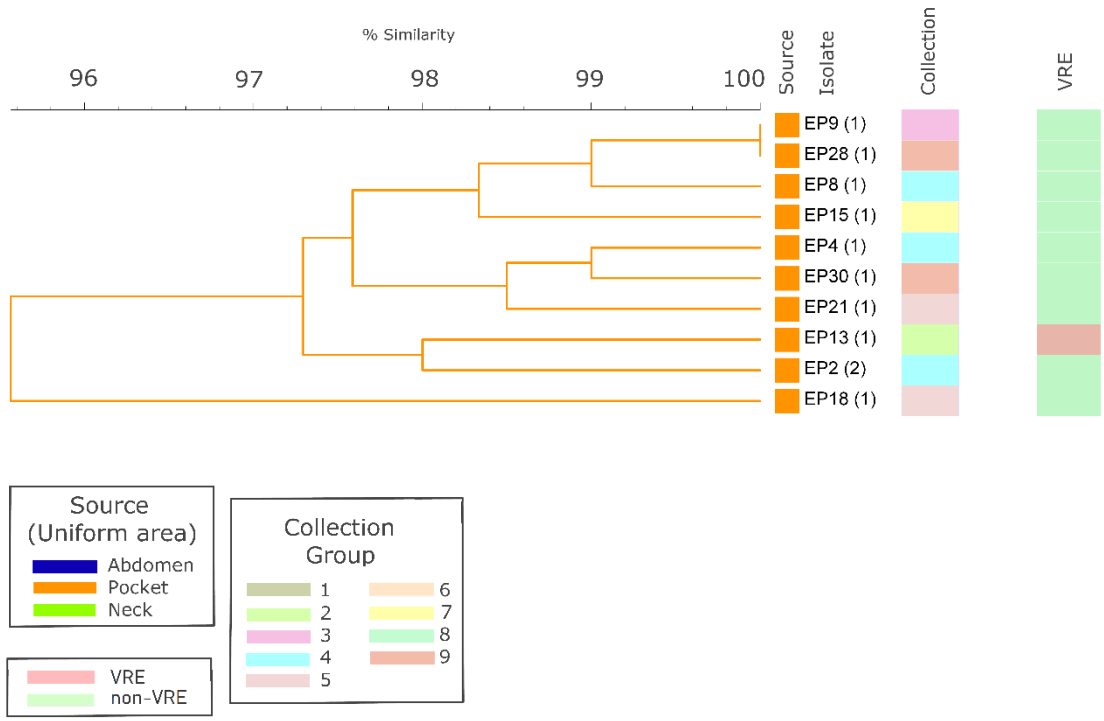

Figure 4.23 – Similarity dendrogram of *Enterococcus* spp. isolates recovered from pocket of post-shift healthcare workers uniforms. Simailrity determined by RAPD testing and subsequent analysis using Bionumerics software. For each isolate RAPD experimental protocols were completed in duplicate and reproducibility assessment conducted, only isolates with reproducibiliy of 99% and above (between two independent replicates) were included in analysis.

4.3.9 Diversity Analysis Using Antibiotic Susceptibility Patterns

For each isolate an antibiogram was produced in *Chapter 3* outlining susceptibility to eight antibiotics. This data represents phenotypic characteristics of each isolate and variation in collective response to the antibiotics can be compared for multiple isolates to determine diversity amongst a bacterial population; i.e. isolates may have different antibiotic susceptibility patterns. Using bionumerics software antibiotic suscpectibility data was used to conduct similarity tests and produce similarity dendrograms. Figure 4.24 and Figure 4.25 show similarity dendrograms for *S. aureus* and *Enterococcus* spp. antibiotic susceptibility data respectively.

For both *S. aureus* and *Enterococcus* spp. isolates, the antibiotic profiles (phenotypic characteristic) show increased diversity amongst isolates when compared to the RAPD profiles (genomic characteristic). *S. aureus* isolates show similarity ranging between <35% and 100% whereas *Enterococcus* spp. isolates show similarity ranging from <40%-100%. There is a large variation in antibiotic profiles in response to the eight antibiotics tested against however there is clear trends with regard susceptibility to a single antibiotic (i.e. clades of isolates all resistant to cefoxitin with an overall similarity). Similarly there is clades of higher similarity based on MDR classification and the source of isolation.

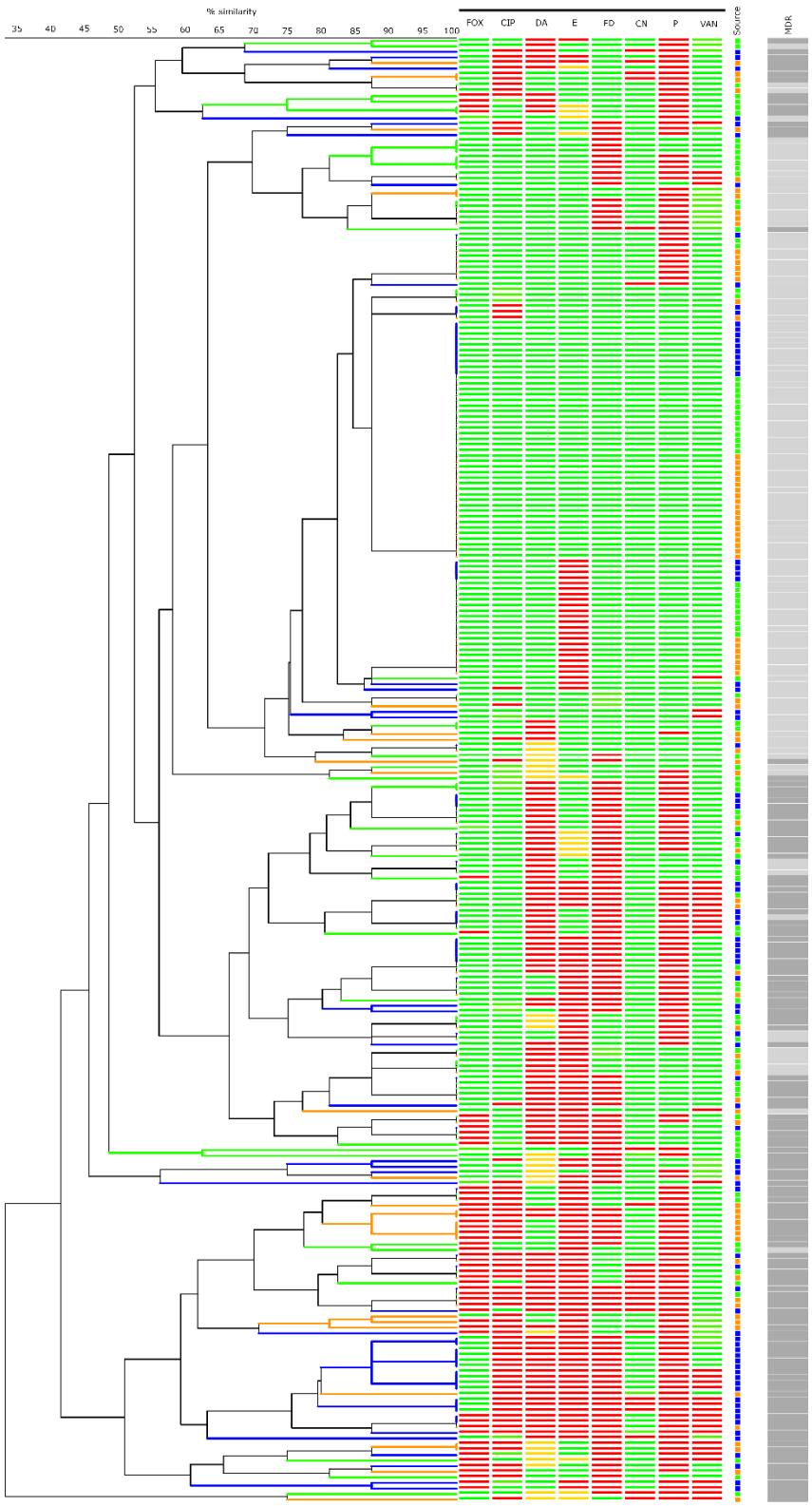

Figure 4.24 – Similarity dendrogram of *Staphylococcus aureus* isolates recovered from post-shift healthcare workers uniforms. Similarity determined by antibiotic susceptibility testsing and subsequent analysis of antibiotic susceptibility profile using Bionumerics software (n=264). Green = susceptible; Red = resistant; Black = Multi-Drug Resistant; Grey = non-Multi-Drug Resistant.

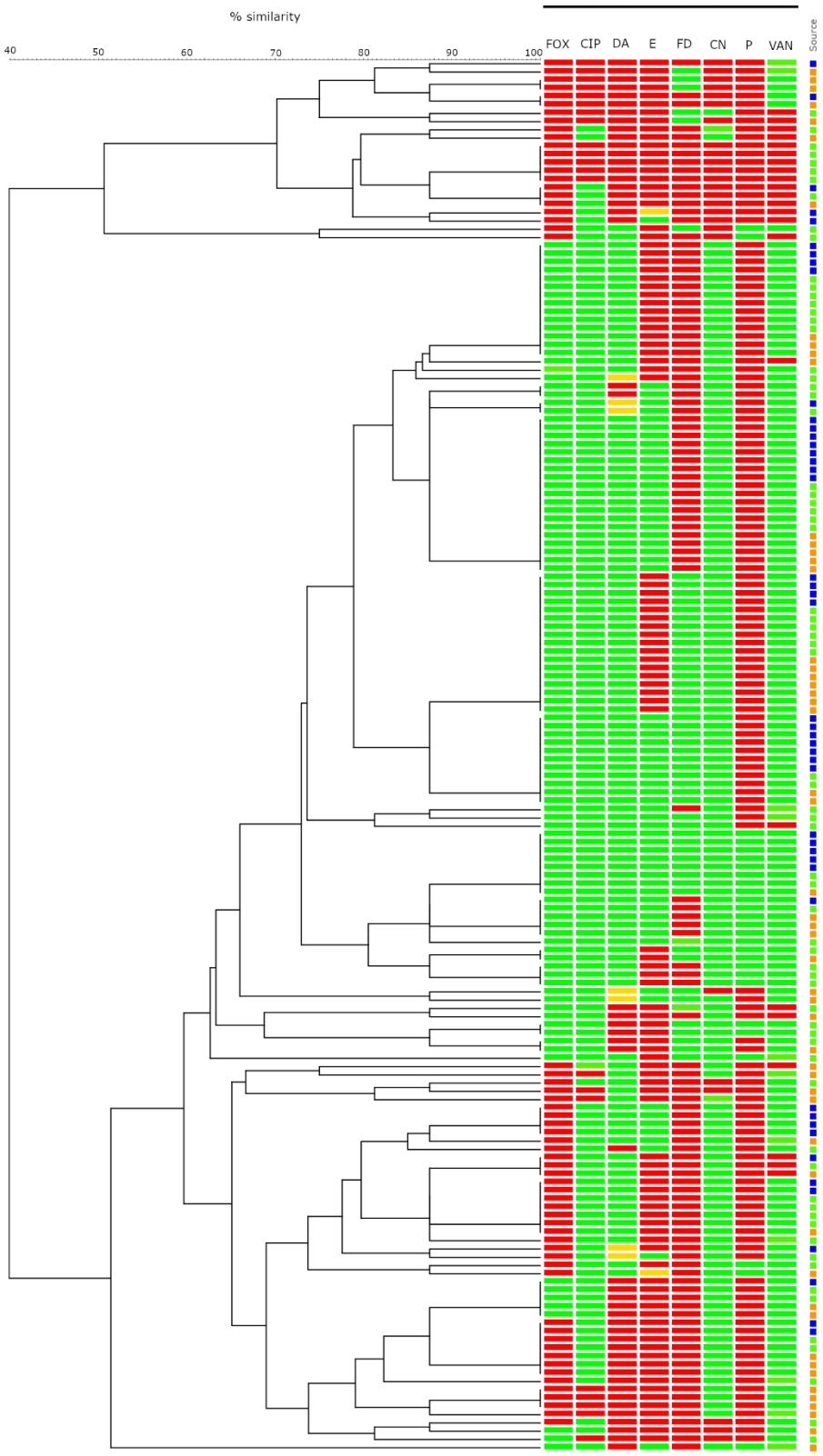

Figure 4.25 – Similarity dendrogram of *Enterococcus* isolates recovered from post-shift healthcare workers uniforms. Similarity determined by antibiotic susceptibility testsing and subsequent analysis of antibiotic susceptibility profile using Bionumerics software (n=169). Green = susceptible; Red = resistant.

4.4 Conclusion

The diversity analysis of *S. aureus* and *Enterococcus* spp. isolated from post-shift healthcare workers uniforms shows that there is variation in phenotypic characteristics and genomic variation at a species/genus level.

4.4.1 RAPD Protocol Development

One of the aims of this work was to develop a protocol/workflow using RAPD to assess genomic diversity amongst a bacterial population. The major issue to overcome is reproducibility of banding patterns produced for each isolate. *S. aureus* ATCC43300, *S. aureus* DSM20231, *E. faecalis* ATCC29212 and *E. faecalis* DSM12956 control isolates were tested for reproducibility using a pre-defined RAPD protocol. Gel electrophoresis reproducibility was 97.5-98.8%, intra-reproducibility was 92.8-98.8% and inter-reproducibility was 86.2-97.8%. The conclusion of these assays is that RAPD is not reproducible. Between 2 independent runs of the same – isolate similarity/reproducibility is as low as 86.2%. This means that due to protocol variability two isolates with the same genome (100% similarity to each other) could be assessed as only 86.2% like each other; i.e. assay does not allow genomic diversity to be assessed accurately.

As RAPD was shown to be variable, additional steps were added to the analysis to allow the user to quantify/quality check the reproducibility of the assay. Duplicate runs of the RAPD protocol were completed for each isolate, the two independent RAPD profiles were used to run a quality check of the data. Each isolate was tested in two independent RAPD runs, the RAPD banding pattern for replicate 1 and replicate 2 were input to bionumerics and a direct comparison for similarity between replicate 1 and replicate 2 was completed. If data was

fully reproducible the software deemed similarity between replicate 1 and 2 (of the same isolate) to be 100%; any protocol variability would be output as diversity between replicate 1 and replicate 2. Based on this data quality control step a reproducible threshold is included to ensure that any diversity seen is representative of genomic diversity rather than variability. Evidence of the importance of including this step to overcome RAPD reproducibility issue is that when no reproducibility threshold is included the dendrogram produced suggests 87% similarity in a large population, however when a threshold of a minimum 95% threshold of reproducibility is included 91% similarity is observed. Further evidence is when a strict 99% reproducibility threshold is included similarity is deemed to be 94%. This trend shows that when reproducibility of a RAPD dataset is included, less variation is observed indicating that if no quality check/reproducibility threshold is included RAPD variation is most likely not representative of diversity between isolates but rather irreproducibility of the protocol.

This data leads to the conclusion that when conducting RAPD assessment [1] independent replicates of samples should be completed, [2] reproducibility between replicates should be assessed/quality checked and [3] only isolates which were shown to be reproducible should be included in data analysis. Only by including these checks can any diversity presented in a dendrogram based on RAPD profiles be considered representative of genomic diversity between isolates. Figure 4.26 shows a data analysis workflow for a single isolate. This workflow is recommended to be included in all RAPD analysis.

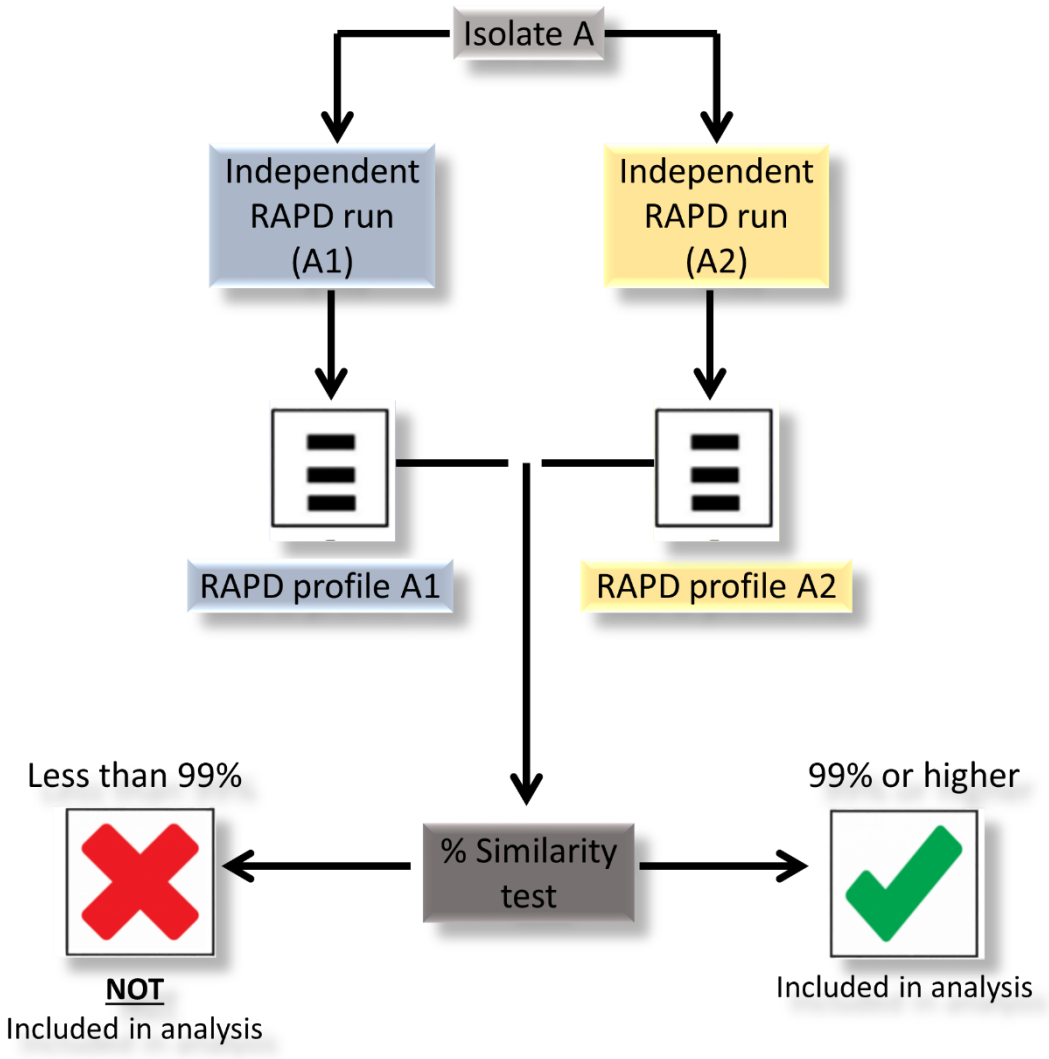

Figure 4.26 – Data analysis workflow for quality check of RAPD data to determine reproducibility. Schematic representative of one isolate – two independent replicates of isolate undergo RAPD protocol producing two RAPD banding patterns/profiles. These profiles are compared in a similarity test to determine reproducibility between replicates. If reproducibility is less than 99% this isolate is not included in the overall data set. If reproducibility is 99% or higher this profile is included in the overall data set.

4.4.2 RAPD Assessment of Diversity of Healthcare Worker Uniform Isolates

RAPD was used to determine genomic diversity amongst *S. aureus* and *Enterococcus* spp. populations. From the bacterial populations and various sub- population (i.e. antibiotic/MDR profile and source of isolation characteristics) it is evident that genomic diversity is present. There is a high level of similarity amongst isolates with many demonstrating >99%-100% similarity to one another. Alternatively, the dendrograms show up to 7% diversity (93-100% similarity) between some isolates of the same species/genus. The dendrograms also display multiple clustering of clades or families within the *S. aureus* and *Enterococcus* spp. populations. Within these clusters there is obvious increased similarity amongst various sub-groups, for example, clustering of all MRSA and non-MRSA isolates – isolates found to be MRSA had increased similarity as a sub-group, this was also the case with non-MRSA, MDR, non-MDR, VRE and non-VRE isolates. Additionally there is increased similarity amongst bacteria isolated in the same collection group. In conclusion, RAPD diversity analysis demonstrates a high level of relatedness amongst *S. aureus* and *Enterococcus* spp. populations isolated from healthcare workers uniforms. Furthermore, there is increased similarity amongst sub-populations based on antibiotic susceptibility and collection points.

The data shows high similarity (93-100%) amongst *S. aureus* bacterial populations. In other published works using RAPD analysis Nikbakht *et al.* (2008) conducted RAPD analysis of MRSA isolated from staff and patients of an Iranian hospital; they found limited cluster groups and 95-100% similarity amongst the population, however it should be noted different RAPD primer sets were used (Nikbakht *et al.*, 2008). Kurlenda *et al.* (2007) assessed 234 MRSA isolates (collected from a Polish hospital over a 7 year period) using the same primer as this work (AP-7) and found only 10 banding patterns, furthermore 84% of isolates

demonstrating the same pattern suggesting a high level of similarity (Kurlenda *et al.*, 2007). Kurlenda *et al.* also suggest primer AP-7 is a low discriminatory RAPD primer. This information supports our findings of high similarity amongst the *S. aureus* isolated from healthcare workers' uniforms. However, theoretically any diversity determined is indicative of genetic diversity and heterogeneity, meaning multiple clones/isolate types are present in the population.

RAPD analysis of *Enterococcus* spp. isolated from healthcare workers' uniforms demonstrated heterogeneity with multiple RAPD profiles present. However, there is a high level of similarity between RAPD banding patterns. A study conducted in a Cork hospital used RAPD to assess VRE isolated from faecal samples, this study also found heterogeneity amongst the population. From a total of 67 samples, 18 distinct RAPD profiles were identified despite other molecular characteristics (virulence gene presence) testing positive for all isolates (Whelton *et al.*, 2016). Lucet *et al*. (2007) used RAPD for assessment of VRE isolated from 39 patients at French university hospital during an outbreak. All isolates demonstrated the same RAPD banding pattern, an expected result from an outbreak. However, in a comparison with VRE isolated from different hospital heterogeneity was evident (assessed by RAPD) amongst isolates from different sources/hospitals – suggesting variation based on source of isolation (Lucet *et al.*, 2007). These data sets identified in the literature support the data from Antrim area hospital with high similarity amongst *Enterococcus* spp. isolates. However, as seen with *S. aureus* isolates with low levels of diversity were detected suggesting multiple isolate types with high similarity amongst the *Enterococcus* spp. population.

4.4.3 Antibiotic Susceptibility Profile Diversity of Healthcare Worker Uniform Isolates

Each isolate was tested against 8 antibiotics using the disc diffusion method following EUCAST guidelines. For a single isolate the result for 8 antibiotics generates an antibiogram profile based on sensitive/resistance patterns. A similarity test of antibiogram profiles was compared using Bionumerics software to produce a dendrogram for *S. aureus* and *Enterococcus* spp. isolates. Giacca *et al.* (1987) first reported using antibiotic profile patterns to produce dendrograms for cluster and diversity comparisons for inexpensive epidemiological analysis of cross-infections in healthcare settings (Giacca *et al.*, 1987).

The antibiogram similarity dendrograms show increased diversity amongst bacterial populations compared to RAPD diversity. *S. aureus* antibiotic profile pattern similarity ranged from 35-100% and *Enterococcus* spp. antibiotic profile pattern similarity ranged from 40-100%. For *S. aureus* isolates classed MDR and/or MRSA demonstrated increased similarity. Similarly VRE and non-VRE isolates had increased similarity in the *Enterococcus* spp. population. Varela *et al.* (2013) also reported multiple antibiotic profiles (7 antibiotics tested) of enterococci from hospital effluent and reported similarity values of 25-100% for 65 isolates, this publication also reported increased diversity between VRE and non-VRE isolates (Varela et al., 2013). Reem *et al.* (2014) also assessed antibiotic profiles for diversity in *S. aureus* (MRSA and MSSA) isolated from high touch surfaces of ophthalmology clinic over a 1-year surveillance study. They also reported diversity by assessing phenotypic antibiotic susceptibility profile along with genotypic diversity (however this was assessed with PFGE rather than RAPD) (Reem et al., 2014). Similarly to the analysis of the Antrim area hospital isolates MRSA and MSSA populations had increased similarity for both phenotypic and genotypic characteristics.

In conclusion the antibiotic profile pattern comparisons suggest bacterial phenotypic diversity suggesting multiple isolate types present in both *S. aureus* and *Enterococcus* spp. populations. This diversity (40-100% similarity) is increased in comparison to diversity determined by RAPD (93-100% similarity). This is an expected result, the RAPD analysis determines genomic diversity based on one primer (i.e. one variable) whereas in the antibiotic profiling the phenotypic response of isolates when challenged with eight antibiotics was used (i.e. eight variables). This would suggest RAPD (using these primers and conditions) does not allow sufficient discriminatory power to effectively assess diversity amongst a large bacterial population. Further evidence of this is different antibiogram profile could have the same or different RAPD profile – therefore not discriminating between clinically different bacteria with regard antibiotic susceptibility. However the antibiogram profiles allows sufficient discriminatory power (evident by high levels of diversity). Furthermore the antibiogram data would be a clinically significant information set, this [A] allows discrimination of isolate types based phenotypic response, and [B] reveals information based on antibiotic resistances and therefore potential treatment options.

However, both RAPD analysis and antibiogram profile comparisons suggest multiple isolate types present in *S. aureus* and *Enterococcus* spp. bacterial populations recovered from healthcare workers uniforms at Antrim Area Hospital (NHSCT).

4.4.4 Clinical Relevance of Bacterial Diversity

Multiple isolates of *S. aureus* and *Enterococcus* spp. presence on healthcare workers uniforms has clinical significance for various reasons. Bacterial diversity at a species/genus level suggests multiple contaminants increasing the bacterial types which could infect patients. Multiple isolate types is also evidence of multiple sources of environmental contamination, leading to healthcare workers uniforms contamination which subsequently act as a potential transmission route of infection to patients. The same RAPD and/or antibiogram profile (i.e. same isolate type) isolated on multiple uniforms, collected on different days is evident in one contaminant/contamination point resulting is subsequent contamination of multiple uniforms over a prolonged timescale (consistent contamination) which could act as vectors of transmission onto patients. An example of this is seen in Figure 4.19 where the same RAPD profile has been isolated from multiple independent uniforms. However, similarity between isolates is increased when isolated from the same collection group which suggests on different days different contaminants are introduced to the hospital environment. In conclusion this suggests continuous contamination of the healthcare environment with multiple isolate types of *S. aureus* and *Enterococcus* spp.

Multple isolate types are present in the bacterial populations therefore the bacteria are different which could translate to differences in phenotype. Pathogenicity variation could be tested by assessing the presence/absence of related virulence genes. Likewise antibiotic resistance could be tested by assessing the presence/absence of relevant antibiotic resistance genes, however the antibiograms represent phenotypic evidence of this variation. With regard infection control practices multiple isolate types potentially could represent variation in bacterial survival mechanisms and resistance to biocides. This would require

testing of disinfection practices and technologies against an array of bacterial and isolate types to accurately determine their potential uses in an infection control practice.

4.4.5 Summary of Findings

- When using RAPD, reproducibility should be assessed to ensure accurate genomic diversity is determined. This can be achieved by including 'quality control' steps of multiple replicates to quantify reproducibility.
- RAPD suggests the biobank of *S. aureus* and *Enterococcus* spp. isolated from healthcare workers' uniforms' at Antrim Area Hospital (NHSCT) contain multiple isolate types.
- Comparison of antibiogram profiles suggests the biobank of *S. aureus* and *Enterococcus* spp. isolated from healthcare workers' uniforms' at Antrim Area Hospital (NHSCT) contain multiple isolate types.
- Multiple isolate types represent consistent contamination of the healthcare environment with an array of contaminants.

Chapter 5

General Discussion

The overall theme of this work was focused on HAIs, the role of contamination on healthcare workers uniforms in the dissemination of HAIs and novel approaches to tackle HAI contamination in the healthcare environment. The presence, survival and persistence of HAIs in the healthcare environment has detrimental implications for public health (mortality and morbidity rates increase) (Magill *et al.*, 2014; Zingg *et al.*, 2015), finance sectors (increased hospital resource use and associated costs) (Lamarsalle *et al.*, 2013) and antimicrobial resistance (Holmes *et al.*, 2016) (increased levels of antimicrobial resistant pathogens and antimicrobial resistance drivers contributing to increased antimicrobial resistance).

In this work we set out to assess levels of bacterial contamination within a real-life setting (Antrim area Hospital) and characterised a proportion of the bacteria recovered. We assessed antibiotic resistance patterns from *S. aureus* and *Enterococcus* spp. recovered from healthcare workers uniforms. We also assessed the efficacy of a novel mode of action disinfectant (GS) in order to determine if it had any utility in an infection control system to prevent surface contamination. The aims of the work can be summarised as follows:

- To assess a novel disinfectant for direct and residual antimicrobial activity against bacteria, biofilm and spores.
- To measure bacterial bioburden contamination on healthcare workers' uniforms as a proxy for environmental contamination, and likely routes of transmission to patients.
- To assess the antimicrobial resistance profiles and population diversity levels of bacteria directly recovered from a healthcare setting.

5.1 Disinfectant Assessment Standards

As discussed in chapter 1, in hospitals disinfection of the hospital environment is a means to reduce bacterial contamination, this has been shown to reduce HAI incidence (White *et al.*, 2008; Simmons *et al.*, 2013). Hospitals use a variety of disinfectants for cleaning of the healthcare environment (Pratt *et al.*, 2007; Lawley *et al.*, 2010; Boyce *et al.*, 2014; Boyce, 2016; Rutala and Weber, 2017). Disinfection conventionally involves physical cleaning purpose and biological cleaning purpose of the environment and microbiological cleaning (removal of microorganisms) (Loveday *et al.*, 2014). Performance of disinfectant (based on efficacy) is the primary consideration in disinfection (Humphreys, 2011) (other contributors include social factors with regard staff compliance to protocols). The European Committee for Standardization (CEN) provides guidelines for validation of disinfection performance – these guidelines are made up of standards with specific pass/fail criteria for performance/effectiveness. For disinfectant performance these guidelines comprises of a 3 phase tiered approach for assessment and validation of a disinfection for use in hospital infection control practices (Hiom *et al.*, 2015). These phases and standards are summarised in Figure 5.1 and Table 5.1. It is important to note that these standards are continually reviewed and updated (Fraise, 2008). The recommended USA system outlined in the United States Pharmacopoeia uses a very similar three tiered approach – this standard is referred to as Section 1072 USP 2015 (Hiom *et al.*, 2015).

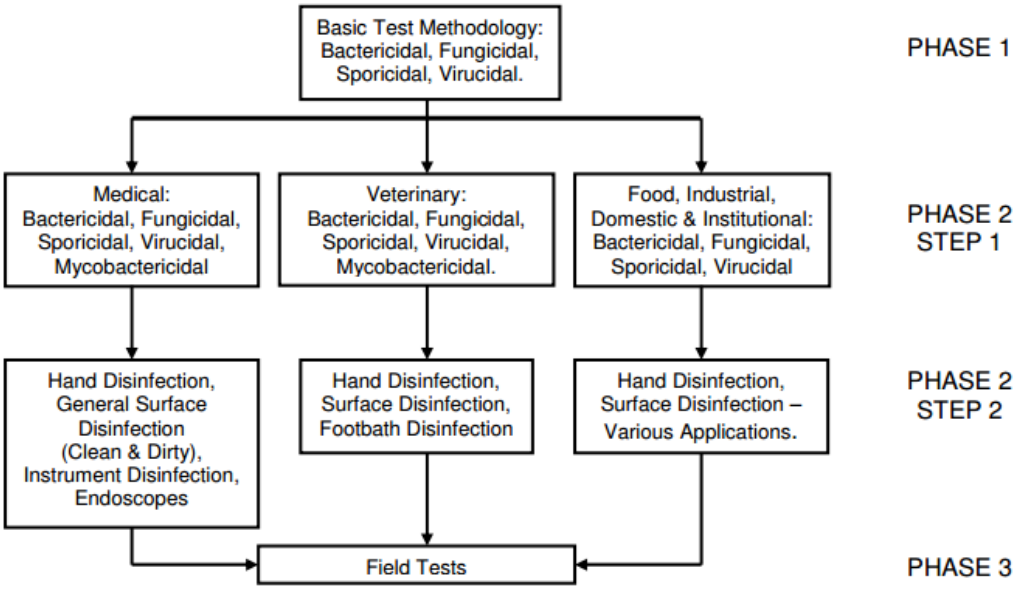

Figure 5.1 – European disinfection testing framework. Overview of processes for assessing the efficacy of disinfectants. Phase 1 and phase 2 assessment are conducted in a laboratory with specific end-point assessment of suspension antimicrobial testing (phase 1) and surface or "intended use" antimicrobial testing (phase 2). Phase 3 testing is conducted on-site of intended use (Humphreys, 2011; European Committee for Standardization, 2015).

Table 5.1 – Overview of tiered testing criteria used to assess performance of disinfectants for use in NHS infection control practices.

Phase	Test	Setting	Standard	Criteria
1	Quantitative suspension test to determine basic biocidal activity.	Laboratory	EN 1040	5 log reduction in ≤5 minutes
2	Quantitative surface test to determine intended use biocidal activity	Laboratory	EN 13697	4 log reduction in ≤5 minutes
3	Intervention study in real life setting to determine before and after benefits	Place of intended use (Hospital)	**None**	**None**

Information collated from EN 1040 and EN 13697.

Phase 1 is the assessment of basic biocidal activity in a laboratory by quantitative suspension tests – For bacteria a 5 Log^{10} reduction in 5 min is required (EN 1040) (The European Committee for Standardization, 2005). For fungi a 4 Log^{10} reduction in 15 min is required (EN 1275) (The European Committee for Standardization, 2006).

In Phase 2 testing, disinfectants are assessed *in vitro*, however tests are designed to assess efficacy under "as intended use" conditions, for example surface tests. These surface tests can vary in design depending on disinfectant characteristics, however normal practice is to assess reduction in viable numbers of microbes following addition of disinfectant on surfaces is determined. To achieve this standard, 4 Log_{10} bacterial reduction in 5 min is required (EN 13697) (Fraise, 2008; The European Committee for Standardization, 2015), 4 Log^{10} fungal reduction in 15 min (EN 1650) (The European Committee for Standardization, 2013) and 3 Log^{10} spore reduction in 60 min (EN 13704) (The European Committee for Standardization). The standards associated with phase 2 do not provide criteria for assessment of novel mode of action disinfectants, such as residual disinfectants or surface acting disinfectants (Hiom *et al.*, 2015).

Phase 3 testing is performed in the actual place of intended use, for example hospitals or care homes. A study in which the product is used in a "real life" scenario is conducted with appropriate controls to assess performance of the disinfectant (Hiom *et al.*, 2015). This ideally provides information on the potential benefits of a given product, however issues include no specific protocol requirements, no standardisation of tests and most importantly there is no end point measurement to effectively assess the performance of products is this scenario. Detailed standards are published by CEN for laboratory testing but no standards

are available for “real-life” testing – each case is primarily judged on a “before” and “after” set of results specific to that setting (Hiom *et al.*, 2015).

5.1.1 Recommendations for New Standard for Phase 3 Testing

Disinfection of the healthcare environment is important to reduce environmental contamination and subsequently reduce infection rates (and associated costs and antibiotic use) (Pratt *et al.*, 2007; Loveday *et al.*, 2014). Whilst testing disinfectant efficacy in the laboratory is important – the absence of standards for assessment in real life settings makes it impossible for the performance of novel, potentially more effective disinfectants to be accurately assessed and subsequently implemented for use in hospital infection control systems (Dancer, 2016; Holmes *et al.*, 2016).

Currently, there is no method of applying information gathered in laboratories to real life settings, for example the question could be asked “does a ~1 Log^{10} reduction in bacteria correlate to a significant reduction in hospital contamination levels and subsequent infection rates?” In order to answer this question the previously discussed standards would require updating to bridge the gap between laboratory and practice. Suggestions would include defining levels of contamination in hospitals (Reynolds *et al.*, 2018) and conducting in place assessments of novel disinfectants to develop an information base. Information required includes current levels of contamination, performance of current disinfectant(s) and performance of novel disinfectants. In addition, monitoring impact of interventions on contamination levels and subsequent impact on infection rates. Such information, if it were generated/available, would lead to accurate efficacy standards required of disinfectants to have a significant impact on contamination levels and infection rates in hospitals; for

example "a 4 Log^{10} reduction of bacteria by a disinfection is a minimum requirement for significant impact in hospitals" – this output is hypothetical but represents an example of potential output to bridge the gap between research and practice. Furthermore, as this information bank grows, such information could be used to create standards for phase 3 testing in the current model (Figure 5.1 and Table 5.1).

5.1.2 Does Goldshield Comply with Current Standards?

Chapter 2 primarily details the testing of GS technologies as an alternative, modern technology disinfectant for use in hospital infection control systems. GS technologies was robustly tested and was shown to be an effective bactericidal product. GS is marketed as a long-lasting disinfectant to prevent contamination. Whilst prolonged activity was evident, this residual bactericidal activity was of low effect (~1 Log^{10} reduction) and the efficacy of the product reduced over time.

In basic antimicrobial suspension tests GS5 achieved 6 Log^{10} reduction of *S. aureus* ATCC43300 in 5 min, thus demonstrating compliance with EN 1040. The current requirements outlined in EN 13697 (surface tests) are a 4 Log^{10} reductions in 5 min. GS5 was tested in surface tests but the results were below the criteria required – Baxa *et al.* (2011) used similar surface tests of GS, and also did not meet the requirements (Baxa *et al.*, 2011). However, GS5 was tested as a residual surface disinfectant, meaning bacteria were applied to clean surfaces post disinfection to assess residual activity of GS. In our testing, the residual activity of GS was up 1 Log^{10} reduction of bacteria. In accordance to the EN 13697 standard GS would not pass and therefore be not suitable for disinfection systems – however, the current standards do not consider novel mode of action disinfectants (such as residual

activity) (Hiom *et al.*, 2015). As standards do not include specifications or considerations for novel/modern technologies it could be argued that they are outdated. For these reasons it is difficult to accurately conclude the potential benefits of 1 Log^{10} residual antibacterial activity (i.e. GS technology). To determine the potential use of GS in infection control systems an intervention study would be required, i.e. phase 3 of EN standards – (see future directions). However, no official standards are available for assessment of phase 3 testing (Reynolds *et al.*, 2018).

5.2 Infection Control Implications on Antimicrobial Resistance: An Alternative Approach to Reduce Antimicrobial Resistance

As discussed in detail in chapter 1, multiple factors contribute to increased infection rates within healthcare settings, one of these factors is microbial contamination of the healthcare environment by potentially pathogenic organisms (Kramer *et al.*, 2006). Many microorganisms can persist on inanimate surfaces for long time periods, and whilst present they pose a risk of (direct or indirect) transmission to susceptible individuals (Ploegmakers *et al.*, 2017). This transmission subsequently results in increased infection rates, increased costs (associated with infections) and increased pressure on antibiotic use – furthermore, increased usage of antibiotics results in increased antimicrobial resistance (Nicolle, 2001; Pratt *et al.*, 2007; Loveday *et al.*, 2014; O'Neill, 2016).

As antibiotic resistance is on the rise, pressure is increasing on alternative approaches to tackle infections (O'Neill, 2016). One approach to the problem is to reduce contamination levels resulting in infection prevention by infection control – the importance of infection control was discussed in chapter 1 (Ploegmakers *et al.*, 2017). This includes methods to

prevent transmission of infectious agents onto susceptible individuals – this is particularly evident in healthcare settings where there is high bacterial contamination levels and transmission routes of these contaminants onto patients (Kramer *et al.*, 2006). Theoretically, reducing the contamination levels in hospitals would reduce HAIs and subsequently reduce pressures on antibiotic use, this in turn would potentially reduce rates of antimicrobial resistance (O'Neill, 2016).

Filice *et al.* (2010) assessed the financial aspects of poor infection control programs in USA and the increased expense resulting by the presence and infection of antibiotic resistance organisms. Infections due to non-resistant *S. aureus* cost $15,923 whereas infections by antibiotic-resistant *S. aureus* (MRSA) cost $34,657 in a USA hospital over a 6 month period. Additional costs were primarily due to increased diagnostic and treatment expenses. The authors concluded that better infection control would reduce the infection rates of MRSA, and thus associated costs and antibiotic use (Filice *et al.*, 2010; O'Neill, 2016).

Infection control is a viable approach to prevent infection. Ignaz Semmelweis, known as the "father of infection control," first implemented infection control measures by recommending healthcare workers clean their hands with chlorine before working with pregnant women susceptible to childbed fever – this is often described as the birth of infection control and was an effective measure to reduce infection rates pre-antibiotics (Best and Neuhauser, 2004; Ploegmakers *et al.*, 2017). Prior antibiotic discovery, infection control was the main objective for infection prevention. Sadly, however antibiotics provided an easy treatment option and due to decades of overuse and misuse, there is now a need for alternative approaches to treat/prevent infections (Landelle *et al.*, 2014; Filice *et al.*, 2010).

The World Health Organisation (WHO) have published a report entitled '*Infection control programmes to contain antimicrobial resistance*' in which they state "*assumption would be that such a programme would decrease antimicrobial-resistant infections proportional to the overall decrease in nosocomial infections*" (Nicolle, 2001). Public Health England regularly publish '*epic: National Evidence-Based Guidelines for Preventing Healthcare-Associated Infections in NHS Hospitals in England*' outlining the importance and key aspects to reduce HAIs and the subsequent reduction on antibiotic use (Pratt *et al.*, 2007; Loveday *et al.*, 2014). Jim O'Neill has also published '*Infection Prevention, Control and Surveillance: Limiting the Development and Spread of Drug Resistance*' as part of his review on antimicrobial resistance (O'Neill, 2016). In this report O'Neill states "*The availability of antimicrobials has shifted the focus from prevention towards treatment*" and "*The only sustainable, long-term solution to the global problems of AMR lies in action to address the 'demand side'*" (O'Neill, 2016) – both points clearly support the alternative approach of preventing infection

In chapter 3 we set out to determine contamination levels of *S. aureus* and *Enterococcus* spp. on healthcare workers' uniforms. *S. aureus* and *Enterococcus* spp. isolated from uniforms were assessed for antibiotic susceptibility. We showed that healthcare workers uniforms become contaminated with highly resistant bacteria during a working shift in a hospital. This contamination confirms environmental contamination (source) and contamination of the uniforms. These contaminants represent potential direct and indirect transmission routes of bacteria to susceptible patients. Furthermore, infections could prove difficult to treat with antibiotics as high levels of antibiotic resistance was evident toward first line antibiotics amongst isolated bacteria. This pressure on antibiotic use could result in increased

antimicrobial resistance. Therefore reducing these contamination levels would theoretically reduce the need for antibiotic use and antimicrobial resistance.

5.3 Concluding Remarks

The work described in this thesis assesses levels of bacterial contamination on healthcare workers uniforms (potential transmission route to patients) and assesses a novel disinfectant to prevent this contamination, i.e. assessed a problem and preliminary assessed possible solution. If GS technology could be shown in practice to reduce contamination levels – this could subsequently lead to reduced HAIs and associated costs. Furthermore if successful, could be considered an alternative to antibiotic use.

In conclusion better infection control reduces [1] infection rates, [2] associated costs, and [3] antibiotic use and [4] antimicrobial resistance.

5.4 Future Directions

The work presented in this thesis highlights a contamination problem of healthcare workers' uniforms in a local hospital which potentially could act as transmission routes onto patients resulting in infection and related issues. Other work assessed a novel approach to prevent this contamination problem occurring – however, this work was conducted in the laboratory. Due to the outdated standards for assessing efficacy of novel disinfectants there are difficulties in applying the information collated in the laboratory to assess potential benefits of GS technology in an infection control practice within a hospital. Therefore, the logical future direction would be to test GS technology in a hospital setting, specifically on uniforms. GS technology has previously been used in a hospital intervention study, by application on high-touch contact surfaces of 18 patient rooms, it was concluded that GS technology could prevent 5-10% of HAI cases compared to the normal infection control practices (Perez *et al.*, 2015). The proposed study would differ in that uniforms would be treated with GS during the laundry processes to coat the uniforms with GS with the aim of preventing contamination rather than surfaces – this is relevant as uniforms are a common indirect transmission route onto patients. A uniform treatment study was described in chapter 3 (Johnston, 2012) however as previously discussed, due to the lacking of sensitivity in recovery of bacteria Johnston (2012) was unable to accurately determine an effect. The protocol described in chapter 3 of this thesis demonstrated high sensitivity of recovery of bacteria therefore would be suitable to accurately quantify any reduction of bacteria numbers.

A proposed intervention would involve enumeration of bacterial contamination on GS-coated healthcare workers uniforms pre-shift and post-shift. The protocols used would mimic those described in chapter 3 where *S. aureus* and *Enterococcus* spp. contamination was assessed on 200 uniforms. The difference would be the inclusion of GS technology in

the laundry of uniforms to allow a “before and after” comparative of the healthcare workers’ uniforms bacterial bioburden in Antrim area hospital. As GS coats all surfaces uniforms material would be coated with the nano-scale ‘bed of nails’ which theoretically prevents contamination. The aims of this study would be to assess contamination levels on healthcare workers uniforms coated with GS and to assess infection rates and antibiotic usage during the testing period.

It would be hypothesised (based on data from this thesis) that contamination levels would be reduced in comparison to the uniforms tested in chapter 3 (i.e. uniforms without GS). Furthermore, it is hypothesised infection rates would reduce and subsequently antibiotic prescribing. Additionally, information gathered from such a study would allow assessment of the value of laboratory testing versus real life testing; i.e. does the laboratory testing presented in this thesis correlate to significant reduction in a real life infection control program.

Chapter 6

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Appendix

PLOS ONE

RESEARCH ARTICLE

Evaluation of bactericidal and anti-biofilm properties of a novel surface-active organosilane biocide against healthcare associated pathogens and *Pseudomonas aeruginosa* biolfilm

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OPEN ACCESS

Citation: Murray J, Muruko T, Gill CIR, Kearney MP, Farren D, Scott MG, et al. (2017) Evaluation of bactericidal and anti-biofilm properties of a novel surface-active organosilane biocide against healthcare associated pathogens and *Pseudomonas aeruginosa* biolfilm. PLoS ONE 12 (8): e0182624. https://doi.org/10.1371/journal.pone.0182624

Editor: Peter Setlow, University of Connecticut, UNITED STATES

Received: October 27, 2016

Accepted: July 21, 2017

Published: August 7, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: JM was supported by a postgraduate research studentship from the Department of Employment and Learning (N Ireland).

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: Goldshield products

Abstract

Healthcare acquired infections (HAI) pose a great threat in hospital settings and environmental contamination can be attributed to the spread of these. De-contamination and, significantly, prevention of re-contamination of the environment could help in preventing/reducing this threat. Goldshield (GS5) is a novel organosilane biocide marketed as a single application product with residual biocidal activity. We tested the hypothesis that GS5 could provide longer-term residual antimicrobial activity than existing disinfectants once applied to surfaces. Thus, the residual bactericidal properties of GS5, Actichlor and Distel against repeated challenge with *Staphylococcus aureus* ATCC43300 were tested, and showed that GS5 alone exhibited longer-term bactericidal activity for up to 6 days on 316I stainless steel surfaces. Having established efficacy against *S. aureus*, we tested GS5 against common healthcare acquired pathogens, and demonstrated that, on average, a 1 $\log^{10}$ bactericidal effect was exhibited by GS5 treated surfaces, although biocidal activity varied depending upon the surface type and the species of bacteria. The ability of GS5 to prevent *Pseudomonas aeruginosa* biofilm formation was measured in standard microtitre plate assays, where it had no significant effect on either biofilm formation or development. Taken together the data suggests that GS5 treatment of surfaces may be a useful means to reducing bacterial contamination in the context of infection control practices.

Introduction

Healthcare acquired infections (HAIs) are directly and indirectly responsible for increased morbidity and mortality rates in hospitals worldwide. In Europe alone there are >4.5 million

and technical information were obtained free of charge from Goldshield Industries (Europe) Ltd, Unit C, Lincoln Lodge Farm, Castlethorpe, MK19 7HJ. Goldshield Industries were not involved in the experimental design, collection, analysis or interpretation of data or in writing the manuscript or decision to publish.

cases annually, which result in >37,000 deaths [1]. A further consequence is the financial burden associated with these infections, measured in terms of increased length of patient stay, decreased bed availability as a result and the extra cost of antibiotic therapy to treat the infection. In the USA alone the total annual expenditure on HAI is estimated to be in excess of $9.8 billion (£6–7 billion) [2], while in Europe a figure of over €7 billion (~£5.5 billion) has been proposed [3]. As a consequence, there is increasing interest from industrial, research and development and healthcare sectors in the development of viable and cost-effective alternative methods of reducing HAI.

Common healthcare associated pathogens include *Staphylococcus aureus* (and predominantly Methicillin resistant *Staphylococcus aureus* (MRSA)), Vancomycin-resistant *Enterococci* (VRE), *Clostridium difficile*, and *Pseudomonas aeruginosa*. Such microorganisms have been shown to survive on inanimate surfaces for extended periods of time—for example *S. aureus* has been shown to survive as long as 6 months [4,5] while *Enterococci* can survive as long as 4 months [6]. *Clostridium difficile* infections (CDI), the most common HAI type in Europe [7] are attributed in part to the persistence of infectious spores on hospital surfaces for up to 5 months [5]. Bacteria capable of forming biofilms, such as *P. aeruginosa* and *S. aureus*, also survive and persist in the environment due to this ability, on top of any intrinsic resistance to antimicrobials [8]. Thus vegetative cells, spores, or biofilms present a threat of infection and indeed a recent report identified biofilm within water taps as the cause of a series of neonatal *P. aeruginosa* infections [9].

Evidence of a direct correlation between environmental contamination and infection rates exists [5,10,11,12] and microbial contamination of the environment has been shown to act as a source of infection that is directly responsible for transmission of organisms to patients [12]. The most problematic areas tend to be high-touch points such as bed rails, door handles, table top surfaces, bedding (mattress), television controls and staff uniforms [13]. Such contaminated surfaces act as a source of direct to patient, and indirect—via healthcare workers/instruments—spread to patients [5,14]. As long as these organisms persist in a hospital or healthcare facility environment they remain a source of infection and therefore, hospitals have implemented revised and improved infection control practices in order to reduce and ideally eradicate environmental microbial contamination. This is achieved primarily by the use of disinfectants and detergents, although the precise disinfectant used will be dependent on multiple factors. For example, areas of high risk such as operating theatres will require multiple cleans per day, whereas patient waiting rooms may be cleaned only once per day. The choice of disinfectant agent is also multifactorial: body fluid spillages will normally require higher level disinfectants than those used in routine cleaning. As a result, hospitals will use a variety of products including ethyl alcohol in hand rubs and gels, Quaternary ammonium compounds (QACs), chlorine-releasing agents and peroxygen sterilants [15]. Nonetheless, current cleaning methods have in several instances been shown to be ineffective. Work by French *et al.* [11] showed that 74% of sites in a London hospital were MRSA positive and when these same sites were retested post-cleaning, all were still contaminated [11]. Recurrence of contamination on surfaces, post disinfection, is therefore a significant issue and this is especially true of high-touch surfaces [16]. Given the available evidence for the ineffectiveness of cleaning and rapid recontamination of surfaces, there is currently much interest in alternative approaches to the problem. The development of intrinsically anti-microbial surfaces that incorporate a variety of agents to kill microbes may be considered a useful strategy. Alternatively, the use of specialised agents that are capable of preventing surface contamination, or that exhibit a residual antimicrobial activity post-disinfection, could be employed, and such products have recently been highlighted as of potential utility in the healthcare setting [17].

One such antimicrobial product is Goldshield, distributed by Goldshield Technologies Ltd. [GS hereinafter]. This is a patented, water soluble organosilane, coupled with a quaternary ammonium compound that is designed to coat surfaces with a protective antimicrobial layer to prevent microbial contamination. The product was originally designed at Emory University, USA and is the subject of three US patents (patent nos. US5,959,014, US6,221,944, and US6,632,805). In this paper we report the bactericidal and anti-biofilm of GS5 technology against 11 common healthcare associated pathogens.

Materials and methods

Chemicals, glassware and media

All glassware was sterilised by soaking overnight in 1% Virkon (Antec, UK) and steam sterilised in an autoclave prior to use. All culture media (Oxoid, UK) was prepared as per the manufacturer's instructions. Phosphate Buffered Saline (Oxoid, UK) was prepared in deionised water and steam sterilised in an autoclave prior to use. Two model surfaces were used. 316l Steel (Aalco, UK) or Formica were cut into 2cm×2cm samples, autoclaved (121˚C for 15 min) and stored in a sealed sterile container prior to use.

Microorganisms

Ten bacterial species were obtained from either the American Type Culture Collection (ATCC) or the Leibniz-Institute DSMZ German Collection of Microorganisms and Cell Cultures (DSMZ). Bacteria included *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* DSM16358, *Mycobacterium smegmatis* DSM43469, *Pseudomonas aeruginosa* DSM3227, *Staphylococcus aureus* (MRSA) ATCC43300, *Staphylococcus aureus* (non-MRSA) DSM20231, *Staphylococcus epidermidis* DSM28319 (all cultured at 37˚C using Nutrient broth/agar), *Enterococcus faecalis* DSM12956 (37˚C using Tryptone soya broth/agar), *Burkholderia multivorans* DSM13243 (28˚C using Nutrient broth/agar) and *Acinetobacter baumannii* DSM30008 (30˚C using Nutrient broth and agar). These were chosen as representative organisms of the type causing HAIs commonly seen in hospitals [18] and included Gram positive organisms, Gram negative organisms and *Mycobacteria*. *Mycobacterium smegmatis* was used as it is a fasting-growing model *Mycobacterium* species [19]. Organisms were stored on Cryobeads (Technical Service Consultants Ltd, UK) at -80˚C and recovered in suitable media when required.

Disinfectant agents

Three disinfectant agents used (GS5, Actichlor and Distel) are classed bactericidal surface disinfectants. The characteristics of these antimicrobial agents are summarised in Table 1. Agents were acquired as full strength concentrate and working stock concentrations were prepared by dilution with deionised water as per the respective manufacturer's instructions.

Direct bactericidal assessment of GS5

To determine directly the bactericidal activity of GS5, a suspension contact time assay was completed; varying concentrations of GS5 were mixed with *S. aureus* ATCC43300, followed by recovery and enumeration of viable cells to determine Log_{10} reduction. 0% (sterile water), 0.25% (v/v), 0.5% (v/v) and 1% (v/v) GS5 dilutions were prepared using sterile water as diluent. A 10 μl aliquot of mid-log *S. aureus* ATCC43300 was mixed with each of the GS5 concentrations and left to stand at room temperature for 5 min. Bacteria were enumerated by dilution plating 0.1ml aliquots onto Nutrient agar in duplicates and incubating at 37˚C for 24 h followed by direct colony counts. Three biologically independent experiments were performed.

Table 1. Antimicrobial products tested.

Agent	Type	Active ingredient	Concentration used*
Goldshield5	Organosilane coupled with Quaternary Ammonium Compound (siQAC)	Trihydroxysilylpropyldimethyloctadecyl ammonium chloride	1:20 dilution
Actichlor	Chlorine-based disinfectant	Sodium dichloroisocyanurate	1:10 dilution
Distel	Quaternary Ammonium Compound	Tertiary alylamine and quaternary ammonium compounds	1:100 dilution

* as per manufacturer's instructions.

https://doi.org/10.1371/journal.pone.0182624.t001

Residual surface activity of disinfectants

To investigate the residual activity of surface disinfectants a protocol was developed from the EN13697 standard and the work of Baxa *et al.* [20]. *Staphylococcus aureus* ATCC43300 (MRSA) and 316l Steel were used. The 316l Steel surface samples were sprayed with either GS5, Actichlor, Distel or sterile water (no treatment control) using a hand spray. The test surfaces were left to dry in the sterile environment of a category 2 cabinet (Biomat). *S. aureus* ATCC43300 was grown to mid log phase of growth (OD_{600} = ~0.48) and diluted 1/100 using sterile PBS (Oxoid, UK). A total of 100 μl of this was added (in 10 μl droplets) to technical triplicate examples of each surface. Bacteria were left on the surfaces for 45 min, and then viable cells recovered in 10 ml of sterile PBS by vortexing for 2 min. Bacteria were enumerated by plating dilution series in duplicate on Nutrient Agar and incubating at 37˚C for 24 h followed by direct colony counts [20]. Following recovery of bacteria from the surfaces each surface was individually washed using sterile PBS, air dried and stored in a sterile storage box. These surfaces were then re-challenged with *S. aureus* ATCC43300 as above. This re-challenge was repeated at 3-day intervals over 15 days. Three biologically independent experiments were performed.

GS5 bactericidal surface testing

A selection of 10 different bacteria, representative of important HAI, were individually tested on 316l Steel and Formica. Testing was performed once to determine the maximum antimicrobial effect for a freshly treated surface. The protocol was as described above, but without re-challenge and only the activity of GS5 was assessed.

Assessment of GS5 efficacy against biofilms

Pseudomonas aeruginosa DSM3227 biofilms were grown in 24-well microtiter plates (4 wells per treatment) and these were stained with 0.1% crystal violet to assess the extent of biofilm growth according to established methods [21,22,23]. To determine efficacy of GS5 against biofilm, Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes, (Thermo Scientific, UK) were pre-treated with either 5% GS5 or sterile water (untreated): wells were soaked with 1 ml of agent for 10 min following which treatment agents were aspirated and plates left to dry in a sterile environment (Biomat category 2 cabinet). An overnight culture of *P. aeruginosa* DSM3227 was diluted 1/100 (using sterile nutrient broth) and microtitre plate wells inoculated with a 1 ml aliquot following which the plates were incubated aerobically at 37˚C. At defined time points (8 h, 12 h, 24 h, 48 h, 72 h and 96 h) biofilm production was assessed. The medium containing planktonic cells was removed and wells stained with 1.5 ml of 0.1% Crystal Violet (Sigma-Aldrich, UK) for 10 min at room temperature. Unbound crystal violet (Sigma-Aldrich,

UK) was removed and stained wells washed twice with 2ml sterile PBS following which bound crystal violet was solubilised using 1.5 ml of 30% Acetic Acid (Thermo Scientific, UK) for 30 min at room temperature. A 1 ml aliquot from each well was transferred to a fresh 24-well microtiter plate and the absorbance of the crystal violet measured at 570nm using a FLUROstar Omega plate reader (BMG LABTECH, Europe). Each experiment was repeated on three separate occasions.

Assessment of GS5 effects on bacterial viability in biofilm

Bacterial viability in biofilms was assessed using the BacLight Live/Dead bacterial viability kit (L-7007; Molecular Probes, Eugene, OR) [24,25]. With Baclight, live cells stain green and dead/damaged cells stain red. A stock solution was prepared by mixing 4 μl of component A (1.67 mM Syto9 plus 1.67 mM propidium iodide), 6 μl of component B (1.67 mM syto9 plus 18.3 mM propidium iodide) and 1ml of sterile water as described by Bauer *et al.* [25]. *P. aeruginosa* DSM3227 biofilm was grown in 4-well Nunc™ Lab-Tek™ II Chamber Slide™ Systems (Thermo Scientific, UK) pre-treated with either 5% GS5 or sterile deionised water. Slides were inoculated with 1 ml of a 1/100 dilution of overnight culture of *P. aeruginosa* and incubated aerobically for 24 h and 48 h at 37˚C. At each time point excess media and planktonic cells were removed and the wells washed with sterile PBS followed by staining with 200 μl BacLight mix and 100 μl of sterile water. Stained slides were incubated in the dark at room temperature for 30 min following which the wells were then washed with sterile PBS and viewed using ×100 oil immersion on a Nikon ECLIPSE E400 (Nikon) microscope utilising a dual-band emission filter (450–490 nm/510–560 nm). Images were generated using NIS-Elements BR (Nikon) software version 3.22.09. Image J software was used to generate composite (red/green) images of the baclight stained biofilms.

Statistical analysis

For bactericidal testing, $\log_{10}$ changes in viable bacterial numbers, compared to untreated controls was determined. The equation Log Reduction LR = $\log_{10}$ ($N_{control}$)–log10 ($N_{treated}$) was used where $N_{control}$ is total recovery of untreated bacteria and $N_{treated}$ is total recovery of treated bacteria. Data was imported to Graphpad Prism 6.01 and charts constructed. Statistical analysis was completed using SPSS v22.

Results

Direct bactericidal assessment of GS5

We firstly wished to determine if GS5 was effective against bacteria in solution, prior to surface testing. We hypothesised that a solution of GS5 at working concentration would exhibit a bactericidal effect against a suspension of bacteria. The direct antibacterial effects of GS5 against *S. aureus* ATCC43300 was assessed using a suspension assay. *S. aureus* ATCC43300 was challenged with increasing concentrations of GS5 to quantify bactericidal activity. GS5 exhibited bactericidal actions at all concentrations after 5min contact time as shown in Fig 1 (0.25% = 4.96 Log_{10} reduction; 0.5% = 5.6 Log_{10} reduction; 1% = 6 Log_{10} reduction (complete kill). Subsequent testing was completed at 5% as per manufacturer's instructions.

Residual activity of surface disinfectants

GS5 is reported to form covalent bonds with surfaces, thereby leaving a nanoscale antimicrobial coating which kills microbes that encounter that surface. This, it is claimed, makes GS5 a more effective product due to its residual antimicrobial activity compared to other

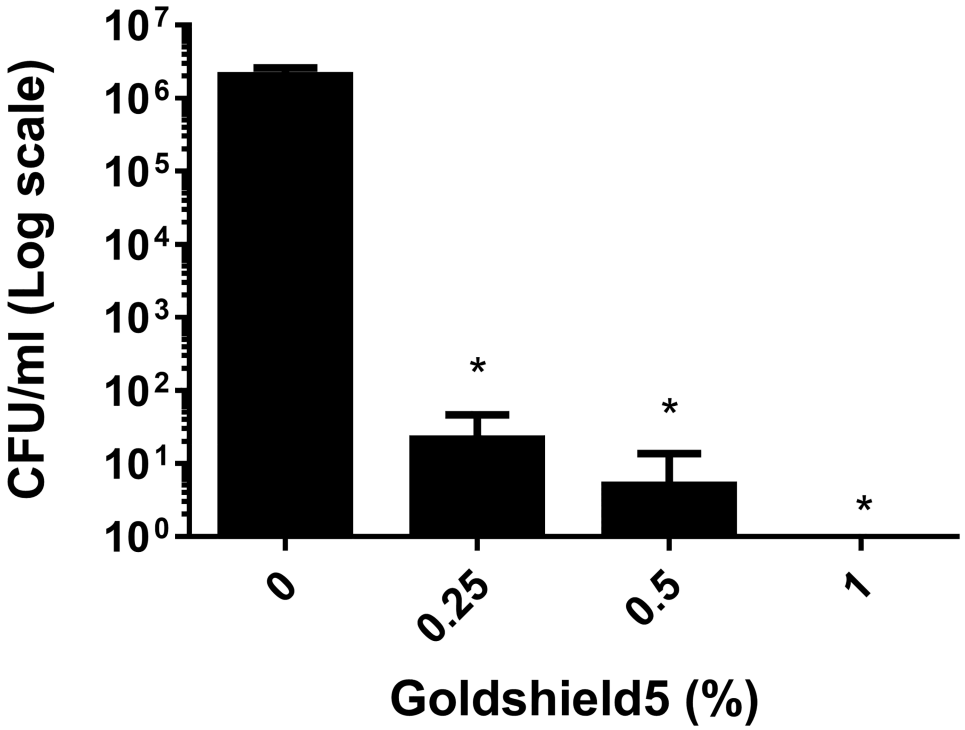

Fig 1. *S. aureus* ATCC43300 survival following suspension test using GS5. ~2×10^6 cfu/ml of *S. aureus* ATCC43300 was challenged with increasing concentrations of GS5. Data represents mean +/- SD of three independent experiments. Statistical analysis by independent T-tests versus Untreated (0%) controls (* = p<0.05, ** = p<0.005, *** = p<0.001).

https://doi.org/10.1371/journal.pone.0182624.g001

disinfectants. We designed an experiment to test this hypothesis by determining the residual antimicrobial effect of GS5, Actichlor and Distel. The bactericidal activity of the three surface disinfectant agents was tested against *S. aureus* ATCC43300 on 316l Steel (Aalco, UK) and residual activity was assessed over 15 days at 3 day intervals. All three products exhibit bactericidal activity on day 0 (Actichlor = 3.75Log$_{10}$ reduction; Distel = 0.54 Log$_{10}$reduction; GS5 = 1.16 Log$_{10}$ reduction). Following subsequent re-challenge of treated surfaces with *S. aureus* ATCC43300 only GS5 showed significant residual bactericidal activity; this residual activity exerted by GS5 was evident for 6 days (Day 3 GS5 = 0.53 Log$_{10}$ reduction; Day 6 GS5 = 0.26 Log$_{10}$ reduction; Fig 2). For subsequent testing of the GS5 product, the maximum effect time point (day 0) was used.

GS5 bactericidal surface testing

Baxa *et al.* [20] suggested that GS5 exhibited variable effect against different bacterial species. We therefore tested GS5 against a range of healthcare acquired infection microorganisms on 316l Steel or Formica to determine bactericidal effect. As hypothesised, GS5 treated surfaces did indeed exhibit a bactericidal effect against all ten tested microorganisms, and this effect was observed on both Formica and steel. The largest bactericidal effect was observed with *Staphylococcus* strains where a >1 log$_{10}$ reduction was observed on 316l Steel (*S. aureus* ATCC43300 = 1.21 Log$_{10}$ reduction; *S. epidermidis* DSM28319 = 1.06 Log$_{10}$reduction) (Table 2). On Formica, however, the GS5 product exhibited a lower bactericidal effect (<0.5 = Log$_{10}$reduction) against both *Staphylococcus* organisms. The average Log$_{10}$ reduction

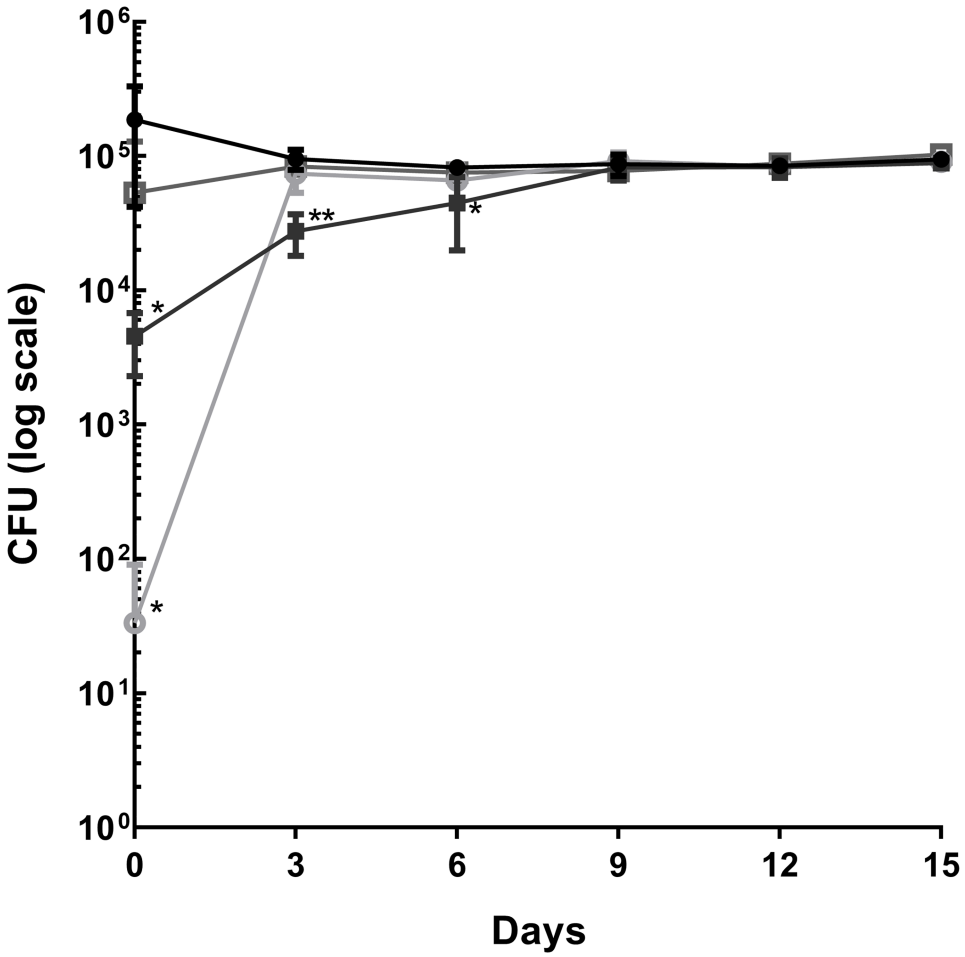

Fig 2. Comparison of residual antimicrobial effects of GS5, Actichlor and Distel on steel surface loaded with *Staphylococcus aureus* ATCC43300. GS5 exhibited prolonged antibacterial activity (6 days) whereas Actichlor and Distel showed no antibacterial activity after day 0. Results are representative of three independent experiments (n = 3; mean+/- SD plotted). Statistical analysis using One way ANOVA and Dunnett's T-test versus Untreated control (* = p<0.05, ** = p<0.005, *** = p<0.001).■ = Goldshield; ● = Untreated control; ○ = Actichlor; □ = Distel.

https://doi.org/10.1371/journal.pone.0182624.g002

on steel surfaces for all bacteria tested was 0.6, whereas the average Log_{10} reduction on Formica was 0.45.

Effect of GS5 on bacterial biofilm formation

Walker *et al.* [9] have demonstrated that biofilm contamination can contribute significantly to outbreaks of healthcare acquired infections. Given the efficacy of GS5 against a range of HAI microbes, we hypothesised that a GS5-treated surface would impede the development of bacterial biofilms. *P. aeruginosa* is a well characterised biofilm former [26], and therefore we pre-treated plastic microtitre plate surfaces with GS5 and assessed the development of *P. aeruginosa* DSM3227 biofilms. The crystal violet staining method provides a quantitative measure of biofilm development/biomass and somewhat unexpectedly our data revealed that GS5 did not appear to inhibit the development of *P. aeruginosa* DSM3227 biofilm in plastic microtiter plates (Fig 3). Having observed that *P. aeruginosa* DSM3227 biofilm development was

Table 2. Log_{10} reductions obtained on GS5 treated surfaces challenged with a variety of microbes.

Organism	Surface	Log_{10} Untreated ± SD	Log_{10} Treated ± SD	Log_{10} change	*p-value*
***Acinetobacter baumannii* DSM30008**	Steel	4.82 ±0.36	4.49 ±0.62	**0.33***	**0.0138**
	Formica	4.25 ±0.04	3.67 ±0.29	**0.58*****	**<0.001**
***Burkholderia multivorans* DSM13243**	Steel	3.90 ±0.14	3.62 ±0.17	**0.28*****	**<0.001**
	Formica	3.94 ±0.05	3.41 ±0.24	**0.53****	**0.0011**
***Enterococcus faecalis* DSM12956**	Steel	5.27 ±0.3	4.8 ±0.08	**0.47**	**0.0623**
	Formica	5.15 ±0.13	4.86 ±0.03	**0.29****	**0.0016**
***Escherichia coli* ATCC25922**	Steel	5.57±0.28	5.32 ±0.33	**0.25****	**0.0018**
	Formica	5.54 ±0.09	5.22 ±0.02	**0.32*****	**<0.001**
***Klebsiella pneumoniae* DSM16358**	Steel	4.30±0.27	3.54 ±0.33	**0.76***	**0.0135**
	Formica	3.94 ±0.05	3.41 ±0.24	**0.53****	**0.0011**
***Mycobacterium smegmatis* DSM43469**	Steel	4.06 ±0.22	3.46 ±0.45	**0.6*****	**<0.001**
	Formica	5.83 ±0.43	5.16 ±0.44	**0.67****	**0.0026**
***Pseudomonas aeruginosa* DSM3227**	Steel	5.09±0.04	4.66±0.29	**0.43****	**0.0017**
	Formica	5.15±0.1	4.63±0.12	**0.52*****	**<0.001**
***Staphylococcus aureus* (MRSA) ATCC43300**	Steel	4.19 ±0.13	2.99 ±0.58	**1.2*****	**<0.001**
	Formica	5.04 ±0.03	4.68 ±0.08	**0.36*****	**<0.001**
***Staphylococcus aureus* (non-MRSA) DSM20231**	Steel	4.57±0.22	3.48±0.27	**1.09*****	**<0.001**
	Formica	5.02±0.23	3.94±0.35	**1.08***	**0.0089**
***Staphylococcus epidermidis* DSM28319**	Steel	3.95 ±0.04	2.88 ±0.05	**1.07****	**0.0047**
	Formica	5.25 ±0.19	4.94 ±0.25	**0.31*****	**<0.001**

Results are representative of three independent experiments (n = 3; mean+/- SD). *p* value calculated using paired T-Test (* = p<0.05, ** = p<0.005, *** = p<0.001).

https://doi.org/10.1371/journal.pone.0182624.t002

apparently unaffected, we assessed bacterial viability within the biofilms using the well-established BacLight staining method. This analysis suggested that a proportion of the bacterial cells were damaged or rendered non-viable when grown on GS5 treated surfaces, but that, critically, a sufficient number of viable/undamaged cells remained (Fig 4) which, we hypothesise are responsible for subsequent biofilm development.

Discussion

Only a single published report exists which details the effects of GS5 used as a surface biocide. GS5 is reported to exert its antimicrobial effect via bonding of the silane end of the molecule to surfaces, following which microbes are drawn onto the hydrocarbon chain. The resultant puncturing of cell membranes and denaturation of proteins is proposed as the cause of cell death [20]. As a covalent bond is formed with the surface it is hypothesised that this mode of action is prolonged creating a 'bactericidal surface'.

When we tested the prolonged activity GS5 exhibited bactericidal activity for 6 days (0.26 log_{10} reduction) whereas the other surface disinfectants tested showed no activity beyond day 0 (Fig 1). In comparison with previous residual testing of the GS5 product by Baxa *et al.* [20], which was completed on fabric swatches rather than on hard surfaces, we observed that residual antimicrobial activity of GS5 was lower (6 days rather than 14 days) [20]. However, the residual antibacterial effect decreased over time to a <1 log_{10} reduction in bacterial numbers, suggesting that GS5 would need regular reapplication and would not be sufficient as a surface disinfectant alone.

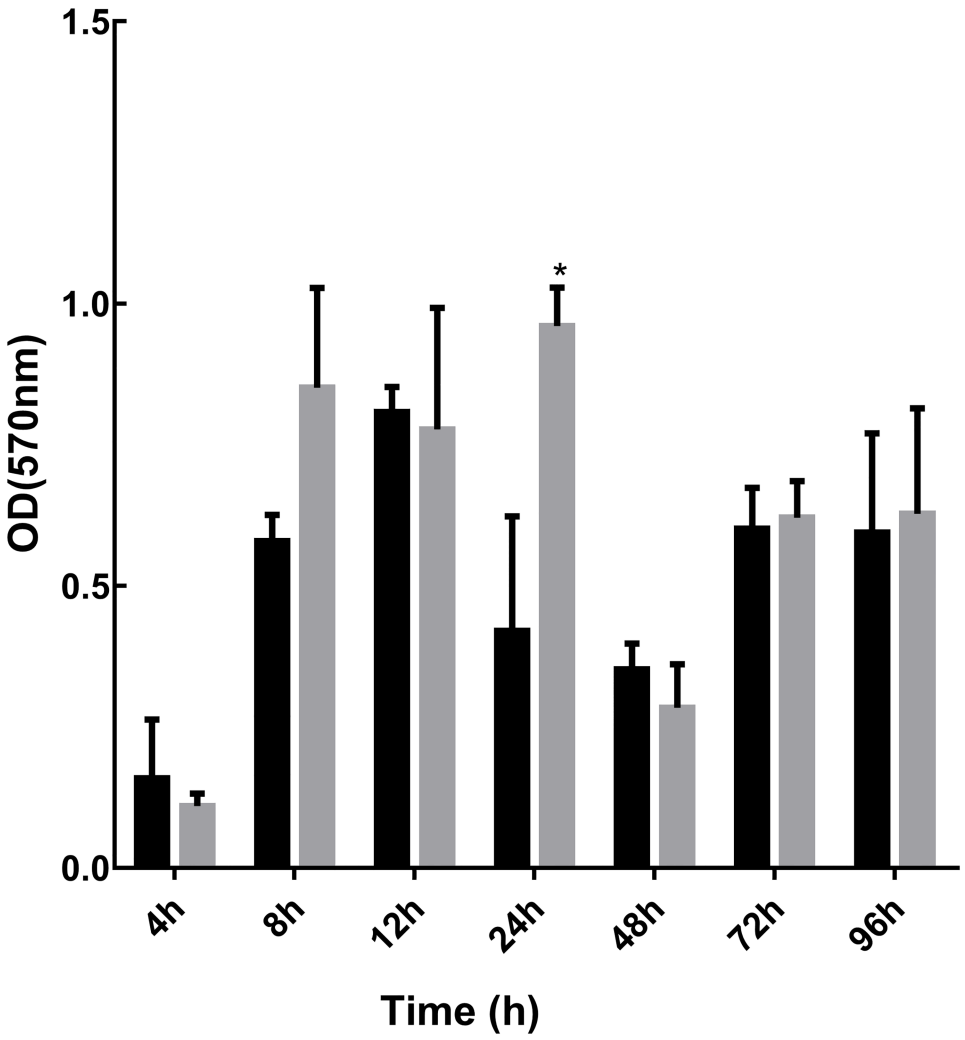

Fig 3. Biofilm development following pre-treatment with GS5. *Pseudomonas aeruginosa* DSM3227 biofilm biomass was assessed by crystal violet staining at various time points and data presented represents mean +/- SD of three independent experiments. Statistical analysis by independent T-tests versus Untreated controls (* = p<0.05, ** = p<0.005, *** = p<0.001). Grey columns representative of pre-treated samples; black bars representative of untreated controls.

https://doi.org/10.1371/journal.pone.0182624.g003

GS5 treated surfaces exhibited bactericidal activity which varied in effectiveness between surface type and bacterial species (Table 1). Thus, bacterial species challenged, in addition to surface type/properties, appears to have a significant influence on the performance of the GS5 product. Surface hydrophobicity, charge and roughness have all been reported as important with respect to performance of biocides [12]. Indeed, variations in the response of bacterial species to disinfectants is evident in the literature with disparate $\log_{10}$ reductions and widely varying minimum inhibitory concentrations (MICs); biocidal resistance is also evident [20,27]. GS5 is said to not induce resistance in microorganisms as a result of its physical mode of action, reported as membrane disruption and protein denaturation. We noted differences between the results of our current work and data reported by Baxa *et al.* [20] who also tested *S. aureus*, *E. coli* and *P. aeruginosa* on steel and Formica. The work of Baxa *et al.* [20] suggested

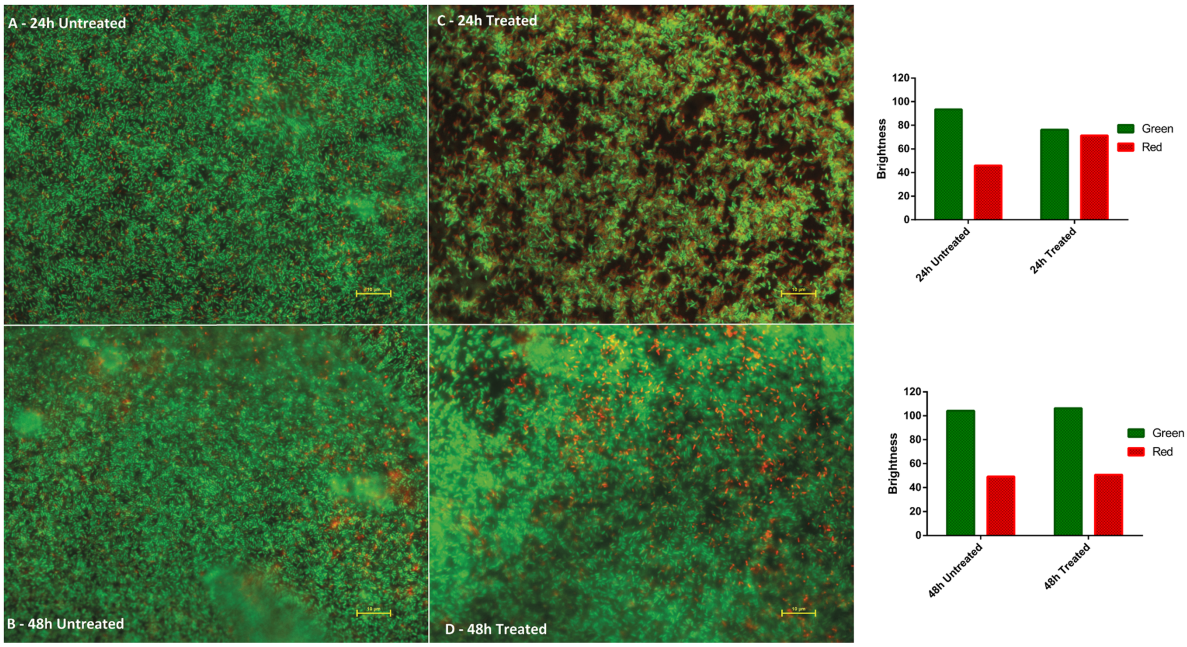

Fig 4. BacLight staining of *P. aeruginosa* DSM3227 biofilm at 24 h and 48 h. Live cells appear green and dead/damaged cells appear red. Images A and B show development of extensive biofilm on untreated surfaces. Image C shows biofilm development on GS5 treated surface with a greater proportion of dead/damaged cells. Image D shows GS5 treated surface biofilm at 48 h: biofilm development and cell viability is similar to the untreated control. Images were obtained ×100 magnification (oil immersion) on a Nikon ECLIPSE E400 (Nikon) microscope utilising a dual-band emission filter (450–490 nm/510–560 nm) and NIS-Elements BR (Nikon) software; composite (red/green) images generated using Image J software. Scale bar = 10 μm. Brightness values were generated for each panel (fig 4 a/b/c/d) using 'imageJ colour histogram analysis' software which converts RBG pixels to brightness values (V = (R+G+B)/3). These red/green brightness values are presented as bar charts to the right of the micrographs.

https://doi.org/10.1371/journal.pone.0182624.g004

that GS5 had greater efficacy against *E. coli* and *P. aeruginosa*, however this observation could be a result of differing surface properties across different types of Steel and Formica used. However, like Baxa *et al.* [20], we have shown that the performance of GS5 against different bacterial species varies considerably, which indicates that the specific type of microbial contaminant will be of greater influence on the effectiveness of GS5, than the actual surface on which it is used.

The ability of HCAI pathogens to adhere, via specific surface proteins to a range of substrates likely to be found in healthcare settings, including polystyrene, has been reported [28]. While biofilms that develop on medical devices such as catheters, chest tubes, prosthetic joints etc. are of concern [29], such medical devices were not the focus of our work. Beyond medical devices, on which biofilms most certainly develop, the contamination of any surface with bacteria in a matrix containing nutrients, will potentially enable development of biofilm. Hospital water systems, from storage to taps, allow biofilm formation and such contamination has been directly linked to adverse health outcomes [9].

Experiments in which plastic surfaces were pre-treated for 10 min with GS5 showed that there was no significant inhibitory effect against *P. aeruginosa* biofilm formation (Fig 3). It is well documented that biofilms exhibit increased resistance to antimicrobials and disinfectants, mainly due to the inability of these molecules to penetrate the biofilm [27]. Given that the GS5-treated plate surfaces would be expected to possess antimicrobial activity, we then considered the viability of cells within developing biofilms. Using BacLight, we observed an initial apparent bactericidal effect on *P. aeruginosa* DSM3227 cells (Fig 4c) as evidenced by a

reduction in biofilm coverage and increased numbers of red stained, damaged, cells at 24 h. This did not translate however, into reduced biofilm formation as measured by crystal violet staining, and indeed later 48 h samples (Fig 4D) showed a well-developed biofilm containing viable cells, similar to that observed in the untreated control (Fig 4B) It is likely, therefore, that residual viable cells maintain the ability to form biofilm and we hypothesise that the cells that are initially damaged by GS5 could actually promote biofilm formation: it has been suggested that dead bacterial cell constituents could comprise a key component of the biofilm or indeed even enhance adhesion and stability of cells, thereby allowing biofilm development [30]. Our data, taken together suggest that GS5 treatment will not significantly inhibit biofilm formation.

Conclusion

Current NHS Infection control practices require that when choosing disinfectants, a 4–5 Log_{10} reduction is required in viable vegetative bacterial cells within a contact/drying time of 10 min, in addition to a spore reduction of 3 Log_{10} within the same period. When tested directly on a suspension of bacterial cells, GS5 achieved a more than 4 Log_{10} reduction with a 5 min contact time however the residual surface active antimicrobial activity of GS5 was much less, at approximately 1 Log_{10} reduction in bacterial numbers. The surface protective effect of GS5 remained for a further 3–6 days without reapplication of the product, however we noted a diminution of the measured Log_{10} reductions over time to a level which was much lower than that required for use in infection control.

Bacteria can form biofilm on surfaces allowing prolonged survival and increased resistance to biocides. Considering the GS5 mode of action we hypothesised a regime where GS5 could be utilised to prevent biofilm formation on surfaces subsequently reducing risk of infection. However GS5 has been shown to possess limited anti-biofilm properties as biofilm production is not impeded on GS5 coated surfaces.

Within the NHS, certain disinfectants (for example, DifficilS) routinely achieve 4 Log_{10} reductions in both vegetative cell and spore numbers within 3–5 min however control of infection is only achievable in practice by using these products in intensive cleaning up to twice daily in a rolling programme of disinfection. Thus, on the basis of the data generated in this work, it appears unlikely, despite modest reductions in bacterial cell viability and evidence for a short lived residual effect, that GS5 would replace current infection control products such as DifficilS or Actichlor in reducing the transmission of HAI pathogens within hospitals and care settings.

Supporting information

S1 Table. Supporting dataset of plate count & A570 data.
(XLSX)

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